PCT/EP2004/003995

Europäisches Patentamt	European Patent Office	Office européen des brevets

REC'D 09 JUN 2004
WIPO PCT

Bescheinigung / Certificate / Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. / Patent application No. / Demande de brevet n°

03076044.1

BEST AVAILABLE COPY

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Europäisches Patentamt | European Patent Office | Office européen des brevets

PCT/EP2004/003995

Anmeldung Nr:
Application no.: 03076044.1
Demande no:

Anmeldetag:
Date of filing: 09.04.03
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Bayer BioScience N.V.
Jozef Plateaustraat 22
9000 Gent
BELGIQUE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Methods and means for increasing the tolerance of plants to stress conditions

In Anspruch genommene Prioriät(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12N15/82

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PT RO SE SI SK TR LI

Methods and means for increasing the tolerance of plants to stress conditions.

Field of the invention.

[1] The present invention relates to the use of poly (ADP-ribose) glycohydrolases in plants to increase the tolerance of plants to adverse growing conditions, including drought, high light intensities, high temperatures, nutrient limitations and the like. Methods and means are provided to produce plants that are tolerant to abiotic stress conditions.

Background to the invention

[2] . Frequently, abiotic stress will lead either directly or indirectly to damage of the DNA of the cells of the plants exposed to the adverse conditions. Genomic damage, if left unrepaired, can lead to cell death. Tolerance to stress conditions exhibited by plants is the result of the ability of the plant cells exposed to the adverse conditions to reduce and/or repair the damage, and to survive.

[3] Plant cells, like other eukaryotic cells, have evolved an elaborate DNA repair system. The activation of poly(ADP-ribose) polymerase (PARP) by DNA strand breaks is often one of the first cellular responses to DNA damage. PARP catalyzes the post-translational modification of proteins by adding successively molecules of ADP-ribose, obtained from the conversion of nicotineamide dinucleotide (NAD), to form multibranched polymers containing up to 200 ADP-ribose residues (about 40 residues in plants). The dependence of poly(ADP-ribose) synthesis on DNA strand breaks, and the presence of PARP in multiprotein complexes further containing key effectors of DNA repair, replication and transcription reactions, strongly suggests that this posttranslational modification is involved in metabolism of nucleic acids, and DNA repair. There are also indications that poly (ADP –ribose) synthesis is involved in regulation of cell cycle and cell death.

[4] Poly (ADP-ribosylation) of proteins is transient in living cells. The poly (ADP-ribose) polymers are rapidly turned over, being converted to free ADP-ribose by the exoglycosidase and endoglycosidase activity of poly (ADP-ribose) glycohydrolase (PARG; E.C.3.2.1.143). The most proximal unit of ADP ribose on the protein acceptor is hydrolysed by the action of another enzyme (ADP-ribosyl protein lyase).

[5] In addition to this positive (DNA-repair associated) effect of PARP on cell survival, there is also a negative effect of PARP. The process of activating PARP upon DNA damage is associated with a rapid lowering of NAD+ levels, since each ADP-ribose unit transferred by PARP consumes one molecule of NAD+. NAD+ depletion in turn results in ATP depletion, because NAD+ resynthesis requires at least (depending on the biosynthesis pathway) three molecules of ATP per molecule of NAD+. Furthermore, NAD+ depletion block glyceraldehyde –3-phosphate dehydrogenase activity, which is required to resynthesize ATP during glycolysis. Finally, NAD+ is a key carrier of electrons needed to generate ATP via electron transport and oxidative phosphorylation.

[6] The physiological consequence of NAD+ and ATP depletion has been established in the context of DNA-damage induced cell death. It has been shown that the completion of apoptosis is absolutely dependent on the presence of ATP and that, in the absence of this nucleotide, the type of cellular demise switches from apoptosis to necrosis. Since the cellular lysis associates with necrosis generates further damage to neighboring cells it is preferable for multicellular organisms to favour apoptotic cell death rather than necrosis.

[7] It is thus very important to consider the delicate balance of positive and negative effects of the poly (ADP ribosyl)ation on the potential of a cell to survive DNA damage.

[8] WO 00/04173 describes methods to modulate programmed cell death (PCD) in eukaryotic cells and organisms, particularly plant cells and plants, by

introducing of "PCD modulating chimeric genes" influencing the expression and/or apparent activity of endogenous poly-(ADP-ribose) polymerase (PARP) genes. Programmed cell death may be inhibited or provoked. The invention particularly relates to the use of nucleotide sequences encoding proteins with PARP activity for modulating PCD, for enhancing growth rate or for producing stress tolerant cells and organisms.

[9] PARG encoding genes have been identified in a number of animals such as *Rattus norvegicus* (Accession numbers: NM_031339, NW_043030, AB019366,), *Mus musculus* (Accession numbers: NT_039598, NM_003631, AF079557), *Homo sapiens* (Accession numbers: NT_017696; NM_003631, AF005043), *Bos taurus* (Accession numbers: NM_174138, U78975) *Drosophila melanogaster* (Accession number: AF079556)

[10] In plants, a poly(ADP-ribose) glycohydrolase has been identified by map-based cloning of the wild-type gene inactivated in a mutant affected in clock-controlled transcription of genes in *Arabidopsis* and in photoperiod dependent transition from vegatative growth to flowering (*tej*). The nucleotide sequence of the gene can be obtained from nucleotide databases under the accession number AF394690 (Panda et al., 2002 Dev. Cell. 3, 51-61).

Summary of the invention

[11] The invention provides a method to produce a plant tolerant to stress conditions comprising the steps of providing plant cells with a chimeric gene to create transgenic plant cells, wherein the chimeric gene comprises the following operably linked DNA fragments: a plant-expressible promoter; a DNA region, which when transcribed yields an ParG inhibitory RNA molecule; and a 3' end region involved in transcription termination and polyadenylation. A population of transgenic plant lines is regenerated from the transgenic plant cell; and a stress tolerant plant line is identified within the population of transgenic plant lines. The ParG inhibitory RNA molecule may comprise a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in the plant cell (the endogenous ParG

gene). The ParG inhibitory RNA molecule may also comprise a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in the plant cell (the endogenous ParG gene). In yet another embodiment, the parG inhibitory RNA may comprise a sense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in the plant cell and an antisense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in the plant cell, wherein the sense and antisense region are capable of forming a double stranded RNA region comprising said at least 20 consecutive nucleotides. The chimeric gene may further comprise a DNA region encoding a self-splicing ribozyme between said DNA region coding for parG inhibitory RNA molecule and the 3' end region. Stress conditions may be selected from heat, drought, nutrient depletion, oxidative stress or high light conditions.

[12] In another embodiment of the invention, a method is provided to produce a plant tolerant to stress conditions comprising the steps of: isolating a DNA fragment of at least 100 bp comprising a part of the parG encoding gene of the plant of interest; producing a chimeric gene by operably linking a plant expressible promoter region to the isolated DNA fragment comprising part of the parG encoding gene of the plant in direct orientation compared to the promoter region; and to the isolated DNA fragment comprising part of the parG encoding gene of said plant in inverted orientation compared to the promoter region, and a 3' end region involved in transcription termination and polyadenylation. These chimeric genes are then provided to plant cells to create transgenic plant cells. A population of transgenic plant lines is regnerated from the transgenic plant cells; and a stress tolerant plant line is identified within the population of transgenic plant lines. The invention also relates to stress tolerant plant cells and plants obtained by this process.

[13] The invention also provides DNA molecules comprising a plant-expressible promoter, operably linked to a DNA region, which when transcribed yields an ParG inhibitory RNA molecule, and to a 3' end region

involved in transcription termination and polyadenylation. The ParG inhibitory RNA molecule may comprise a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in the plant cell (the endogenous ParG gene). The ParG inhibitory RNA molecule may also comprise a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in the plant cell .(the endogenous ParG gene). In yet another embodiment, the parG inhibitory RNA may comprise a sense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in the plant cell and an antisense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in the plant cell, wherein the sense and antisense region are capable of forming a double stranded RNA region comprising said at least 20 consecutive nucleotides. The chimeric gene may further comprise a DNA region encoding a self-splicing ribozyme between said DNA region coding for parG inhibitory RNA molecule and the 3' end region.

[14] In yet another embodiment, the invention relates to plant cell comprising the DNA molecule of the invention and plants consisting essentially of such plant cells, as well as to processes for producing stress tolerant plants, comprising the step of further crossing such plants with another plant. Seeds and propagating material of such plants comprising the chimeric genes of the invention are also provided.

Brief description of the figures.

[15] Figure 1. Schematic representation of the poly-ADP ribose polymeratization / depolymerization cycle by the action of PARP/PARG in a eukaryotic cell.

[16] Figure 2. Diagram of the NAD+ and ATP content of *Arabidopsis* lines under high light stress. Dark boxes represent NAD content under high light conditions expressed as percentage of the value for NAD content determined

under low light conditions. Light boxes represent ATP content under high light conditions expressed as percentage of the value for ATP content determined under low light conditions.

[17] Figure 3. Diagram of the NAD+ and ATP content of corn lines under nitrogen depletion stress. Dark boxes represent NAD content while gight boxes represent ATP content.

Detailed description of preferred embodiments

[18] The invention is based, on the one hand, on the demonstration that cells from stress resistant plant lines comprising a chimeric gene reducing the PARP gene expression, exhibited a higher NAD/ATP content under adverse conditions than cells from untransformed plant lines. On the other hand, it has been observed that silencing of the expression of PARG encoding gene in tobacco using a transient silencing RNA vector based on satellite viruses resulted in a similar phenotype as that observed for silencing of PARP encoding gene using the same silencing system.

[19] Although not intending to limit the invention to a specific mode of action, it is expected that silencing of PARG gene expression results in a similar phenotype as silencing of PARP gene expression for the following reasons. As can be seen from Figure 1, polymerization of ADP ribose catalyzed by PARP, consuming NAD, is followed by depolymerization of poly ADP ribose, catalyzed by PARG. Poly ADP ribosylation of the PARP protein itself results in inactivation of the PARP protein. The speed at which the ADP ribose polymerization / depolymerization cycle occurs in plant cells, leading to NAD depletion and consequently ATP depletion, can be slowed down or stopped by reduction of the PARP gene expression or of the enzymatic activity of PARP. As a result, plant cells, and plants comprising such cells are more resistant to adverse conditions. The data provided here indicate that a similar effect can be obtained through slowing down or stopping the cycle by reduction of the PARG gene expression or PARG activity.

[20] The invention relates to reduction of plant cell death in response to adverse environmental conditions, and consequently to enhanced stress resistance, by altering the level of expression of ParG genes, or by altering the activity or the apparent activity of PARG proteins in that plant cell. Conveniently, the level of expression of ParG genes may be controlled genetically by introduction of chimeric genes altering the expression of ParG genes, or by altering the endogenous PARG encoding genes.

[21] In one embodiment of the invention, a method for producing plants tolerant to stress conditions or adverse growing conditions is provided comprising the steps of:

- providing plant cells with a chimeric gene to create transgenic plant cells, wherein the chimeric gene comprises the following operably linked DNA fragments:
    - a plant-expressible promoter;
    - a DNA region, which when transcribed yields a ParG inhibitory RNA molecule;
    - a 3' end region involved in transcription termination and polyadenylation;
- regenerating a population of transgenic plant lines from said transgenic plant cell; and
- identifying a stress tolerant plant line within said population of transgenic plant lines.

[22] As used herein "a stress tolerant plant" or " a plant tolerant to stress conditions or adverse growing conditions" is a plant (particularly a plant obtained according to the methods of the invention), which, when subjected to adverse growing conditions for a period of time, such as but not limited to drought, high temperatures, limited supply of nutrients (particularly nitrogen), high light intensities, grows better than a control plant not treated according to the methods of the invention. This will usually be apparent from the general appearance of the plants and may be measured e.g., by increased biomass production, continued vegetative growth under adverse conditions or higher seed yield. Stress tolerant plant have a broader growth spectrum, i.e. they

are able to withstand a broader range of climatological and other abiotic changes, without yield penalty. Biochemically, stress tolerance may be apparent as the higher NAD^{+}-NADH /ATP content and lower production of reactive oxygen species of stress tolerant plants compared to control plants under stress condition. Stress tolerance may also be apparent as the higher chlorophyll content, higher photosynthesis and lower chlorophyll fluorescence under stress conditions in stress tolerant plants compared to control plants under the same conditions.

[23] It will be clear that it is also not required that the plant be grown continuously under the adverse conditions for the stress tolerance to become apparent. Usually, the difference in stress tolerance between a plant or plant cell according to the invention and a control plant or plant cell will become apparent even when only a relatively short period of adverse conditions is encountered during growth.

[24] As used herein, a "ParG inhibitory RNA molecule" is an RNA molecule that is capable of decreasing the expression of the endogenous PARG encoding genes of a plant cell, preferably through post-transcriptional silencing. It will be clear that even when a ParG inhibitory RNA molecule decreases the expression of a PARG encoding gene through post-transcriptional silencing, such an RNA molecule may also exert other functions within a cell, such as e.g. guiding DNA methylation of the endogenous ParG gene, again ultimately leading to decreased expression of the PARG encoding gene. Also, expression of the endogenous PARG encoding genes of a plant cell may be reduced by transcriptional silencing, e.g., by using RNAi or dsRNA targeted against the promoter region of the endogenous ParG gene.

[25] As used herein, a "PARG encoding gene" or a "ParG gene" is a gene capable of encoding a PARG (poly ADP ribose glycohydrolase) protein, wherein the PARG protein catalyzes the depolymerization of poly ADP-ribose,

by releasing free ADP ribose units either by endoglycolytic or exoglycolytic action.

[26] PARG encoding genes may comprise a nucleotide sequence encoding a protein comprising the amino acid sequence of SEQ ID No 1 (*Arabidopsis thaliana*) or of SEQ ID No 2 (*Solanum tuberosum*) or parts thereof, such as a DNA fragment comprising the nucleotide sequence of SEQ ID No. 3 or SEQ ID 4.

[27] However, it will be clear that the skilled person can isolate variant DNA sequences from other plant species, by hybridization with a probe derived from the above mentioned PARG encoding genes from plant species, or even with a probe derived from the above mentioned PARG encoding genes from animal species. To this end, the probes should preferably have a nucleotide sequence comprising at least 40 consecutive nucleotides from the coding region of those mentioned PARG encoding genes sequences, preferably from the coding region of SEQ ID No 3 or SEQ ID No 4. The probes may however comprise longer regions of nucleotide sequences derived from the ParG genes, such as about 50, 60, 75, 100, 200 or 500 consecutive nucleotides from any of the mentioned ParG genes. Preferably, the probe should comprise a nucleotide sequence coding for one of the highly conserved regions of the catalytic domain, which have been identified by aligning the different PARG proteins from animals. These regions are also present in the identified PARG protein from *Arabidopsis thaliana* and comprise the amino acid sequence LXVDFANXXXGGG (corresponding to SEQ ID No 1 from the amino acid at position 252 to the amino acid at position 264; X may be any amino acid) LXVDFANXXXGGGXXXXGXVQEEIRF (corresponding to SEQ ID No 1 from the amino acid at position 252 to the amino acid at position 277) or LXVDFANXXXGGGXXXXGXVQEEIRFXXXPE (corresponding to SEQ ID No 1 from the amino acid at position 252 to the amino acid at position 282), TGXWGCGXFXGD (corresponding to SEQ ID No 1 from the amino acid at position 449 to the amino acid at position 460) or TGXWGCGAFXGDXXLKXXXQ (corresponding to SEQ ID No 1 from the amino acid at position 449 to the amino acid at position 468). Other conserved

regions have the amino acid sequence DXXXRXXXXAIDA (corresponding to SEQ ID No 1 from the amino acid at position 335 to the amino acid at position 344) or REXXKAXXGF (corresponding to SEQ ID No 1 from the amino acid at position 360 to the amino acid at position 369) or GXXXXSXYTGY (corresponding to SEQ ID No 1 from the amino acid at position 303 to the amino acid at position 313). Hybridization should preferably be under stringent conditions.

[28] "Stringent hybridization conditions" as used herein mean that hybridization will generally occur if there is at least 95% and preferably at least 97% sequence identity between the probe and the target sequence. Examples of stringent hybridization conditions are overnight incubation in a solution comprising 50% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared carrier DNA such as salmon sperm DNA, followed by washing the hybridization support in 0.1 x SSC at approximately 65 °C. Other hybridization and wash conditions are well known and are exemplified in Sambrook et al, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989), particularly chapter 11.

[29] Alternatively, ParG encoding genes or parts thereof may also be isolated by PCR based techniques, using as primers oligonucleotides comprising at least 20 consecutive nucleotides from a nucleotide sequence of the mentioned PARG encoding genes or the complement thereof. Such primers may comprise a nucleotide sequence encoding a conserved region, as mentioned above, or be complementary to such a nucleotide sequence. Oligonucleotides which may be used for that purpose may comprise the nucleotide sequence of either or SEQ ID No.5, SEQ ID No 6., SEQ ID No. 7 or SEQ ID No. 8.

[30] Specific PCR fragments from ParG genes may e.g., be obtained by using combinations of the oligonucleotides having the nucleotide sequence of SEQ ID No. 5 and SEQ ID No 6 using e.g., *Arabidopsis* genomic DNA or

cDNA as a template DNA, or by using combinations of the oligonucleotides having the nucleotide sequence of SEQ ID No. 7 and SEQ ID No 8 using e.g., potato genomic DNA or cDNA as a template DNA, under stringent annealing conditions.

[31] The isolated sequences may encode a functional PARG protein or a part thereof. Preferably the isolated sequences should comprise a nucleotide sequence coding for one or more of the highly conserved regions from the catalytic domain of PARG proteins as mentioned elsewhere.

[32] However, for the purpose of the invention is not required that the isolated sequences encode a functional ParG protein nor that a complete coding region is isolated. Indeed, all that is required for the invention is that a chimeric gene can be designed or produced, based on the identified or isolated sequence of the endogenous ParG gene from a plant, which is capable of producing a ParG inhibitory RNA. Several alternative methods are available to produce such a ParG inhibitory RNA molecule.

[33] In one embodiment, the ParG inhibitory RNA molecule encoding chimeric gene is based on the so-called antisense technology. In other words, the coding region of the chimeric gene comprises a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the endogenous ParG gene of the plant cell or plant, the expression of which is targeted to be reduced. Such a chimeric gene may be conveniently constructed by operably linking a DNA fragment comprising at least 20 nucleotides from the isolated or identified ParG gene, or part of such a gene, in inverse orientation, to a plant expressible promoter and 3'end formation region involved in transcription termination and polyadenylation. It will be immediately clear that there is no need to know the exact nucleotide sequence or complete nucleotide sequence of such a DNA fragment from an isolated ParG gene.

[34] In another embodiment the ParG inhibitory RNA molecule encoding chimeric gene is based on the so-called co-suppression technology. In other

words, the coding region of the chimeric gene comprises a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the endogenous ParG gene of the plant cell or plant, the expression of which is targeted to be reduced. Such a chimeric gene may be conveniently constructed by operably linking a DNA fragment comprising at least 20 nucleotides from the isolated or identified ParG gene, or part of such a gene, in direct orientation, to a plant expressible promoter and 3'end formation region involved in transcription termination and polyadenylation. Again it is not required to know the exact nucleotide sequence of the used DNA fragment from the isolated ParG gene.

[35] The efficiency of the above mentioned chimeric genes in reducing the expression of the endogenous ParG gene may be further enhanced by inclusion of DNA elements which result in the expression of aberrant, unpolyadenylated ParG inhibitory RNA molecules. One such DNA element suitable for that purpose is a DNA region encoding a self-splicing ribozyme, as described in WO 00/01133.

[36] The efficiency or the above mentioned chimeric genes in reducing the expression of the endogenous ParG gene of a plant cell may also be further enhanced by including into one plant cell simultaneously a chimeric gene as herein described encoding a antisense ParG inhibitory RNA molecule and a chimeric gene as herein described encoding a sense ParG inhibitory RNA molecule, wherein said antisense and sense ParG inhibitory RNA molecules are capable of forming a double stranded RNA region by base pairing between the mentioned at least 20 consecutive nucleotides, as described in WO 99/53050.

[37] As further described in WO 99/53050, the sense and antisense ParG inhibitory RNA regions, capable of forming a double stranded RNA region may be present in one RNA molecule, preferably separated by a spacer region. The spacer region may comprise an intron sequence. Such a chimeric gene may be conveniently constructed by operably linking a DNA fragment comprising at least 20 nucleotides from the isolated or identified endogenous

ParG gene, the expression of which is targeted to be reduced, in an inverted repeat, to a plant expressible promoter and 3'end formation region involved in transcription termination and polyadenylation. To achieve the construction of such a chimeric gene, use can be made of the vector described in WO 02/059294

[38] An embodiment of the invention thus concerns a method for obtaining a stress tolerant plant line comprising the steps of

- providing plant cells with a chimeric gene to create transgenic plant cells, wherein the chimeric gene comprises the following operably linked DNA fragments:
    - a plant-expressible promoter;
    - a DNA region, which when transcribed yields a ParG inhibitory RNA molecule comprising a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in said plant cell; or
    - a DNA region, which when transcribed yields a ParG inhibitory RNA molecule comprising a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in said plant cell; or
    - a DNA region, which when transcribed yields a ParG inhibitory RNA molecule comprising a sense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in said plant cell and an antisense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in said plant cell, wherein said sense and antisense region are capable of forming a double stranded RNA region comprising said at least 20 consecutive nucleotides.
    - a 3' end region involved in transcription termination and polyadenylation;
- regenerating a population of transgenic plant lines from said transgenic plant cell; and

- identifying a stress tolerant plant line within said population of transgenic plant lines.

[39] As used herein "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region which is functionally or structurally defined, may comprise additional DNA regions etc.

[40] It will thus be clear that the minimum nucleotide sequence of the antisense or sense RNA region of about 20 nt of the ParG coding region may be comprised within a larger RNA molecule, varying in size from 20 nt to a length equal to the size of the target gene.

[41] The mentioned antisense or sense nucleotide regions may thus be about from about 21 nt to about 5000 nt long, such as 21nt, 40 nt, 50 nt, 100nt, 200 nt, 300nt, 500nt, 1000 nt, 2000 nt or even about 5000 nt or larger in length.

[42] Moreover, it is not required for the purpose of the invention that the nucleotide sequence of the used inhibitory ParG RNA molecule or the encoding region of the chimeric gene, is completely identical or complementary to the endogenous ParG gene the expression of which is targeted to be reduced in the plant cell. The longer the sequence, the less stringent the requirement for the overall sequence identity is. Thus, the sense or antisense regions may have an overall sequence identity of about 40 % or 50% or 60 % or 70% or 80% or 90 % or 100% to the nucleotide sequence of the endogenous ParG gene or the complement thereof. However, as mentioned antisense or sense regions should comprise a nucleotide sequence of 20 consecutive nucleotides having about 100% sequence identity

to the nucleotide sequence of the endogenous ParG gene. Preferably the stretch of about 100 % sequence identity should be about 50, 75 or 100 nt.

[43] For the purpose of this invention, the "sequence identity" of two related nucleotide sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e. a position in an alignment where a residue is present in one sequence but not in the other is regarded as a position with non-identical residues. The alignment of the two sequences is performed by the Needleman and Wunsch algorithm (Needleman and Wunsch 1970) Computer-assisted sequence alignment, can be conveniently performed using standard software program such as GAP which is part of the Wisconsin Package Version 10.1 (Genetics Computer Group, Madison, Wisconsin, USA) using the default scoring matrix with a gap creation penalty of 50 and a gap extension penalty of 3.

[44] It will be clear that whenever nucleotide sequences of RNA molecules are defined by reference to nucleotide sequence of corresponding DNA molecules, the thymine (T) in the nucleotide sequence should be replaced by uracil (U). Whether reference is made to RNA or DNA molecules will be clear from the context of the application.

[45] It will also be clear that chimeric genes capable of producing inhibitory ParG genes for a particular ParG gene in a particular plant variety or plant species, may also be used to inhibit ParG gene expression in other plant varieties or plant species. Indeed, when sufficient homology exists between the ParG inhibitory RNA region and the ParG gene, or when the ParG genes share the same stretch of 19 nucleotides, expression of those other genes will also be down-regulated.

[46] In view of the potential role of ParG in nucleic acid metabolism, it may be advantageous that the expression of the endogenous ParG gene by the ParG inhibitory RNA is not completely inhibited. Downregulating the expression of a particular gene by gene silencing through the introduction of a

chimeric gene encoding ParG inhibitory RNA will result in a population of different transgenic lines, exhibiting a distribution of different degrees of silencing of the ParG gene. The population will thus contain individual transgenic plant lines, wherein the endogenous ParG gene is silenced to the required degree of silencing. A person skilled in the art can easily identify such plant lines, e.g. by subjecting the plant lines to a particular adverse condition, such a high light intensity, oxidative stress, drought, heat etc. and selecting those plants which perform satisfactory and survive best the treatment.

[47] As used herein, the term "promoter" denotes any DNA which is recognized and bound (directly or indirectly) by a DNA-dependent RNA-polymerase during initiation of transcription. A promoter includes the transcription initiation site, and binding sites for transcription initiation factors and RNA polymerase, and can comprise various other sites (e.g., enhancers), at which gene expression regulatory proteins may bind.

[48] The term "regulatory region", as used herein, means any DNA, that is involved in driving transcription and controlling (i.e., regulating) the timing and level of transcription of a given DNA sequence, such as a DNA coding for a protein or polypeptide. For example, a 5' regulatory region (or "promoter region") is a DNA sequence located upstream (i.e., 5') of a coding sequence and which comprises the promoter and the 5'-untranslated leader sequence. A 3' regulatory region is a DNA sequence located downstream (i.e., 3') of the coding sequence and which comprises suitable transcription termination (and/or regulation) signals, including one or more polyadenylation signals.

[49] In one embodiment of the invention the promoter is a constitutive promoter. In another embodiment of the invention, the promoter activity is enhanced by external or internal stimuli (inducible promoter), such as but not limited to hormones, chemical compounds, mechanical impulses, abiotic or biotic stress conditions. The activity of the promoter may also regulated in a temporal or spatial manner (tissue-specific promoters; developmentally regulated promoters).

[50] For the purpose of the invention, the promoter is a plant-expressible promoter. As used herein, the term "plant-expressible promoter" means a DNA sequence which is capable of controlling (initiating) transcription in a plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell, i.e., certain promoters of viral or bacterial origin such as the CaMV35S (Hapster et al., 1988), the subterranean clover virus promoter No 4 or No 7 (WO9606932), or T-DNA gene promoters but also tissue-specific or organ-specific promoters including but not limited to seed-specific promoters (e.g., WO89/03887), organ-primordia specific promoters (An et al., 1996), stem-specific promoters (Keller et al., 1988), leaf specific promoters (Hudspeth et al., 1989), mesophyl-specific promoters (such as the light-inducible Rubisco promoters), root-specific promoters (Keller et al.,1989), tuber-specific promoters (Keil et al., 1989), vascular tissue specific promoters (Peleman et al., 1989), stamen-selective promoters (WO 89/10396, WO 92/13956), dehiscence zone specific promoters (WO 97/13865) and the like.

[51] Methods for the introduction of chimeric genes into plants are well known in the art and include *Agrobacterium*-mediated transformation, particle gun delivery, microinjection, electroporation of intact cells, polyethyleneglycol-mediated protoplast transformation, electroporation of protoplasts, liposome-mediated transformation, silicon-whiskers mediated transformation etc. The transformed cells obtained in this way may then be regenerated into mature fertile plants.

[52] The transgenic plant cells and plant lines according to the invention may further comprise chimeric genes which will reduce the expression of PARP genes as described in WO 00/04173. These further chimeric genes may be introduced e.g. by crossing the transgenic plant lines of the current invention with transgenic plants containing PARP gene expression reducing chimeric genes. Transgenic plant cells or plant lines may also be obtained by introducing or transforming the chimeric genes of the invention into transgenic plant cells comprising the PARP gene expression reducing chimeric genes or

vice versa. Alternatively, the PARP and PARG inhibitory RNA regions may be encoded by one chimeric gene and transcribed as one RNA molecule.

[53] The chimeric genes of the invention (or the inhibitory RNA molecules corresponding thereto) may also be introduced into plant cells in a transient manner, e.g using the viral vectors, such as viral RNA vectors as described in WO 00/63397 or WO 02/13964.

[54] Having read this specification, it will be immediately clear to the skilled artisan, that mutant plant cells and plant lines, wherein the PARG activity is reduced may be used to the same effect as the transgenic plant cells and plant lines described herein. Mutants in ParG gene of a plant cell or plant may be easily identified using screening methods known in the art, whereby chemical mutagenesis, such as e.g., EMS mutagenesis, is combined with sensitive detection methods (such as e.g., denaturing HPLC). An example of such a technique is the so-called "Targeted Induced Local Lesions in Genomes" method as described in McCallum et al, Plant Physiology 123 439-442 or WO 01/75167. However, other methods to detect mutations in particular genome regions or even alleles, are also available and include screening of libraries of existing or newly generated insertion mutant plant lines, whereby pools of genomic DNA of these mutant plant lines are subjected to PCR amplification using primers specific for the inserted DNA fragment and primers specific for the genomic region or allele, wherein the insertion is expected (see e.g. Maes et al., 1999, Trends in Plant Science, 4, pp 90-96).

[55] Plant cell lines and plant lines may also be subjected to mutagenesis by selection for resistance to ParG inhibitors, such as gallotannines . (Ying, et al. (2001). Proc. Natl. Acad. Sci. USA 98(21), 12227-12232; Ying, W., Swanson, R.A. (2000). NeuroReport 11(7), 1385-1388.

[56] Thus, methods are available in the art to identify plant cells and plant lines comprising a mutation in the ParG gene. This population of mutant cells or plant lines can then be subjected to different abiotic stresses, and their

phenotype or survival can be easily determined. Additionally, the NAD and/or the ATP content of the stressed cells can be determined and compared to results of such determinations of unstressed cells. In stress tolerant cells, the reduction of NAD content under stress conditions should when compared with unstressed cells, should be lower than for corresponding control cells.

[57] It is also an object of the invention to provide plant cells and plants containing the chimeric genes or the RNA molecules according to the invention. Gametes, seeds, embryos, either zygotic or somatic, progeny or hybrids of plants comprising the chimeric genes of the present invention, which are produced by traditional breeding methods are also included within the scope of the present invention.

[58] The plants obtained by the methods described herein may be further crossed by traditional breeding techniques with other plants to obtain stress tolerant progeny plants comprising the chimeric genes of the present invention.

[59] The methods and means described herein are believed to be suitable for all plant cells and plants, both dicotyledonous and monocotyledonous plant cells and plants including but not limited to cotton, Brassica vegetables, oilseed rape, wheat, corn or maize, barley, alfalfa, peanuts, sunflowers, rice, oats, sugarcane, soybean, turf grasses, barley, rye, sorghum, sugar cane, vegetables (including chicory, lettuce, tomato, zucchini, bell pepper, eggplant, cucumber, melon, onion, leek), tobacco, .potato, sugarbeet, papaya, pineapple, mango, *Arabidopsis thaliana*, but also plants used in horticulture, floriculture or forestry (poplar, fir, eucalyptus etc.).

[60] The following non-limiting Examples describe method and means for increasing stress tolerance in plants according to the invention.

[61] Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second

Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. Other references for standard molecular biology techniques include Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, NY, Volumes I and II of Brown (1998) Molecular Biology LabFax, Second Edition, Academic Press (UK). Standard materials and methods for polymerase chain reactions can be found in Dieffenbach and Dveksler (1995) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, and in McPherson at al. (2000) PCR - Basics: From Background to Bench, First Edition, Springer Verlag, Germany.

[62] Throughout the description and Examples, reference is made to the following sequences:

[63] SEQ ID N°1: amino acid sequence of the ParG protein from *Arabidopsis thaliana*.

[64] SEQ ID N°2: amino acid sequence of part of the ParG protein from *Solanum tuberosum*.

[65] SEQ ID N°3: nucleotide sequence encoding the ParG protein from *Arabidopsis thaliana*.

[66] SEQ ID N°4: nucleotide sequence encoding the part of the ParG protein from *Solanum tuberosum*.

[67] SEQ ID N°5: nucleotide sequence of an oligonucleotide primer suitable for PCR amplification of part of a ParG protein encoding DNA fragment.

[68] SEQ ID N°6: nucleotide sequence of an oligonucleotide primer suitable for PCR amplification of part of a ParG protein encoding DNA fragment.

[69] SEQ ID N°7: nucleotide sequence of an oligonucleotide primer suitable for PCR amplification of part of a ParG protein encoding DNA fragment.

[70] SEQ ID N°8: nucleotide sequence of an oligonucleotide primer suitable for PCR amplification of part of a ParG protein encoding DNA fragment.

[71] SEQ ID N°9: nucleotide sequence of the T-DNA vector containing the ParG expression reducing chimeric gene based on the *Arabidopsis* ParG gene sequence.

[72] SEQ ID N°10: amino acid sequence of conserved sequence 1 of PARG proteins.

[73] SEQ ID N°11: amino acid sequence of conserved sequence 2 of PARG proteins.

[74] SEQ ID N°12: amino acid sequence of conserved sequence 3 of PARG proteins.

[75] SEQ ID N°13: amino acid sequence of conserved sequence 4 of PARG proteins.

[76] SEQ ID N°14: amino acid sequence of conserved sequence 5 of PARG proteins.

Examples

Example 1. Analysis of the influence of stress on energy production efficiency of transgenic stress tolerant plant lines containing PARP gene expresssion reducing chimeric genes.

[77] Hypocotyls of transgenic *Brassica napus* plants comprising PARP gene expression reducing chimeric genes as described in WO 00/04173 were cultivated for 5 days on a growth medium. Explants were then transferred to liquid medium comprising 30 mg/L aspirin or acetylsalicylic acid (resulting in oxidative stress conditions) for one day. In control experiments, hypocotyls of non-transgenic *Brassica napus* plants N90-740 were cultivated on the same growth medium and then incubated for one day in liquid medium comprising 30 mg/L aspirin. In addition, hypocotyls of both the transgenic lines and the control line were cultivated on the same growth medium without aspirin.

[78] After the cultivation period, the ATP content of 125 explants was determined for each experiment. Additionally, the oxygen consumed in 3 hours by 125 explants was determined. The results are summarized in Table 1. The standard error of the mean was less than 6%. Whereas, the ratio of moles ATP per mg consumed oxygen in the control plants decreased in the control plants when oxidative stress was applied, the same ratio in the stress tolerant transgenic plant lines actually increased under stress conditions, and was considerably higher (about 24%) than in the control plants. The stress resistant transgenic lines thus maintained an constant energy production efficiency, whereas the control lines exhibited an decreased energy production efficiency. In addition, superoxide production, expressed as a percentage of superoxide production in control plants not subjected to the oxidative stress, did not increase in stress tolerant plants subjected to stress conditions.

[79] Table 1. Influence of stress on energy production efficiency of 5 days cultured Brassica napus hypocotyl explants.

Plant line	Stress	moles ATP per 125 explants	O_2 mg/L consumed in 3 hrs by 125 explants	moles ATP / mg consumed O_2	Superoxide production
N90-740 (control)	None	12.4×10^{-7}	2.96	4.19×10^{-7}	100%
	30mg/L aspirin	13.2×10^{-7}	4.06	3.25×10^{-7}	167%
Transgenic line	None	9.3×10^{-7}	2.33	3.99×10^{-7}	108%
	30mg/L aspirin	11.4×10^{-7}	2.82	4.04×10^{-7}	100%

[80] In another experiment, the NAD+ and ATP content of 4 different transgenic *Arabidopsis* lines comprising PARP gene expression reducing chimeric genes as described in WO 00/04173 were determined under high and low light conditions, and compared to the values obtained for a non transformed control line under the same conditions. The 4 different lines exhibited different degrees of stress resistance as exhibited e.g. by their ability to withstand heat and/or drought conditions. The values obtained for the NAD and ATP contents under high light stress are expressed as a percentage of the values for the NAD and ATP contents under low light conditions, and are plotted in Figure 2.

[81] The results show that high light stress leads to a significant NAD reduction in control plant cells and in the transgenic plant line which is the least stress resistant. The more stress resistant the transgenic plant lines are, the less signicifant the NAD reduction is under high light stress conditions.

[82] In another experiment, the NAD+ and ATP content of a segregating population resulting from a cross between transgenic corn lines comprising PARP gene expression reducing chimeric genes as described in WO 00/04173 and an untransformed corn line, were determined under conditions of nutrient (nitrogen) depletion, and compared to the values obtained for a non transformed control line under the same conditions. Figure 3 is a graphic representation of the of the obtained results. Hemizygous and azygous lines were discriminated by verification for the presence of the selectable marker gene. The NAD and ATP content was significantly higher in the hemizygous, stress tolerant plants than in the untransformed control plants or the azygous plants.

Example 2. Construction of ParG gene expression reducing chimeric genes.

[83] To reduce the expression of the PARG gene e.g. in *Arabidopsis* and related plants, a chimeric gene was constructed which is capable expressing a dsRNA comprising both a sense and antisense region which can form a double stranded RNA. Such dsRNA is very effective in reducing the expression of the genes with which is shares sequence homology, by post-transcriptional silencing. The chimeric gene comprises the following DNA fragments:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment comprising 163 bp from the ParG gene from *Arabidopsis thaliana* in direct orientation (Genbank Accession number AF394690 from nucleotide position 973 to 1135);
- A DNA fragment encoding intron 2 from the pdk gene from Flaveria;
- The DNA fragment comprising 163 bp from the ParG gene from *Arabidopsis thaliana* in inverted orientation (Genbank Accession number AF394690 from nucleotide position 973 to 1135)
- A fragment of the 3' untranslated end from the octopine synthetase gene from *Agrobacterium tumefaciens*.

[84] This chimeric gene was introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene providing resistance to the herbicide phosphinotricin.

[85] To reduce the expression of the PARG gene e.g. in potatoes and related plants, a chimeric gene is constructed which is capable expressing a dsRNA comprising both a sense and antisense region of a cDNA sequence from potato, that is capable of encoding a protein having high sequence identity with the N-terminal part of the *Arabidopsis* PARG protein. The chimeric gene comprises the following DNA fragments:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment comprising a sequence of at least 100 bp from ParG homologue from *Solanum tuberosum* in direct orientation (Genbank Accession number BE340510);
- A DNA fragment encoding intron 2 from the pdk gene from Flaveria;
- The DNA fragment comprising the sequence of at least 100 bp from ParG homologue from *Solanum tuberosum* in inverted orientation (Genbank Accession number BE340510);
- A fragment of the 3' untranslated end from the octopine synthetase gene from *Agrobacterium tumefaciens*

[86] This chimeric gene is introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene providing resistance to the herbicide phosphinotricin.

Example 3. Analysis of transgenic plant lines comprising ParG gene expression reducing chimeric genes.

[87] The chimeric genes of Example 2 are introduced into *Arabidopsis* or potato respectively, by Agrobacterium mediated transformation.

[88] The population of obtained transgenic lines is subjected to the following stress conditions, together with control plants:

- Increased heat for a period of days (greenhouse) or hours (in vitro)
- Drought for a period of days
- High light conditions for a period of days
- Nutrient depletion

[89] Individual plant lines surviving well the above mendioned stress conditions are selected.

[90] The NAD content and ATP content for the above mentioned plants is determined under control and stress conditions.

Example 4. Quantitative determination of NAD, ATP and superoxide radicals in plant cells.

[91] Quantification of ATP in plant tissues was done basically as decribed by Rawyler et al. (1999), Plant Physiol. 120, 293-300. The assay was used for the determination of the ATP content of hypocotyl explants that were cultured for 4-5 days on A2S3 medium or 2 weeks old *in vitro* cultured *Arabidopsis* plants. All manipulations are performed on crushed ice unless otherwhise indicated.

[92] ATP extraction

- Freeze plant material with liquid nitrogen
  - 100 hypocotyl explants
  - ± 700mg *Arabidopsis* plants (roots + shoots) (about 32-37 18-days old C24 plants)
- Put frozen hypocotyls in mortar and add 6ml of 6% perchloric acid.
- Extraction can be done at room temperature using a pestle. After extraction, put samples as soon as possible on ice.
- Centrifuge at 24,000g (Sorvall, SS34 rotor at 14,000rpm) for 10min. at

4°C.

- The supernatant is neutralized with 5M K_2CO_3 (add 350µl of 5M K_2CO_3 to 3ml of supernatant).
- $KClO_4$ is removed by spinning as described above.

[93] Quantitative bioluminescent determination of ATP

- The ATP bioluminescent assay kit from Sigma is used (FL-AA).
- Dilute extract 6000 x (about 6 mL extract from which 100µl is taken, that is diluted 1000 times) The dilutions are made with the 'ATP assay mix dilution buffer' (FL-AAB) of the ATP bioluminescent assay kit
- The amount of light that is produced is measured with the TD-20/20 luminometer of Turner Designs (Sunnyvale, USA).
- Standard curve: disolve ATP standard of kit (FL-AAS) in 10ml of water ($2x10^{-6}$ moles)

[94] Quantification of NAD+ and NADH in plant tissues was performed, essentially as described by Karp et al. (1983) or Filipovic et al. (1999) on the following plant material:

Brassica napus: 150 5-days cultured hypocotyl explants/sample

Arabidopsis: 1000mg 18-days old in vitro grown plants (shoots + roots)/sample (corresponds to ±60 C24 plants)

[95] *Assay* solution

(A) For measuring NADH: 25mM potassium phosphate buffer pH7
0.1mM DTT
3µM FMN (Fluka, 83810)
30µM n-decanal (Sigma, D-7384)

(B) For measuring NAD^+ + NADH:
idem as for measuring NADH alone
+ 2µg/mL alcohol dehydrogenase (Roche, 102 717)

[96] Extraction

- Freeze with liquid nitrogen
- Put frozen plant material in cooled mortar (cooled at –20°C) and add 5mL extraction buffer
- Grind material using a pestle
- Centrifuge at 24 000g (Sorvall, SS34 rotor at 14 000rpm) for 15 minutes at 4°C
- Take 1 mL of supernatant for analysis

[97] Assay

NADH

- 390µL of assay solution A
- + 10µL extract
- + 2µL NAD(P)H:FMN oxidoreductase
- + 100µL luciferase solution

NAD^+ + NADH

- 390µL of assay solution B
- + 10µL extract
- 2 minutes at room temperature
- + 2µL NAD(P)H:FMN oxidoreductase
- + 100µL luciferase solution

The amount of light that is produced is measured with the TD-20/20 luminometer of Turner Designs (Sunnyvale, USA)

NADH-standard

NADH stock solution: 1mM (7.1mg/10mL H_2O)

NADH: disodium salt, Roche, 107 735

Dilution series in 10mM potassium phosphate buffer pH7: (10^{-2}); $5x10^{-3}$; $2x10^{-3}$; 10^{-3}; $5x10^{-4}$

Add 10µL of dilutions in 390µL of assay solution A and perform reaction

Make standard curve

[98] Superoxide radicals production was measured by quantifying the reduction of XTT as described in De Block and De Brouwer (2002) Plant Physiol. Biochem. 40, 845-852

[99] BRASSICA NAPUS

[100] Media and reaction buffers

Sowing medium (medium 201):

Half concentrated Murashige and Skoog salts

2% sucrose

pH 5.8

0.6% agar (Difco Bacto Agar)

250mg/l triacillin

Callus inducing medium A2S3:

MS medium, 0.5g/l Mes (pH 5.8), 3% sucrose, 40mg/l adenine-SO_4, 0.5% agarose, 1mg/l 2,4-D, 0.25mg/l NAA, 1mg/l BAP, 250mg/l triacillin

Incubation medium:

25mM K-phosphate buffer pH5.8

2% sucrose

1 drop Tween20 for 25ml medium

Reaction buffer:

50mM K-phosphate buffer pH7.4

1mM sodium,3'-{1-[phenylamino-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) = XTT (bts, Germany, cat n° 2525)

1 drop Tween20 for 25ml buffer

[101] Sterilization of seeds - pregermination of seeds - growing of the seedlings. Seeds are soaked in 70% ethanol for 2 min, then surface-sterilized for 15 min in a sodium hypochlorite solution (with about 6% active chlorine) containing 0.1% Tween20. Finally, the seeds are rinsed with 1l of sterile tap water. Incubate seeds for at least one hour in sterile tap water (to allow diffusion from seeds of components that may inhibit germination). Seeds are put in 250ml erlenmeyer flasks containing 50ml of sterile tap water (+ 250mg/l triacillin). Shake for

about 20 hours. Seeds from which the radicle is protruded are put in Vitro Vent containers from Duchefa containing about 125ml of sowing medium (10 seeds/vessel, not too many to reduce loss of seed by contamination). The seeds are germinated at ±24°C and 10-30:Einstein/$s^{-1}m^{-2}$ with a daylength of 16h.

[102] Preculture of the hypocotyl explants and induction of stress

- 12-14 days after sowing, the hypocotyls are cut in about 7-10mm segments.
- The hypocotyl explants (25 hypocotyls/Optilux Petridish, Falcon S1005, Denmark) are cultured for 5 days on medium A2S3 at 25°C (at 10-30□Einstein/$s^{-1}m^{-2}$).

[103] XTT-assay

- Transfer 150 hypocotyl explants to a 50ml Falcon tube.
- Wash with reaction buffer (without XTT).
- Add 20mL reaction buffer + XTT.
  (explants have to be submerged, but do not vacuum infiltrate)
- Incubate in the dark at 26°C for about 3hours
- Measure the absorption of the reaction medium at 470nm

[104] *ARABIDOPSIS THALIANA*

[105] Media and reaction buffers

Plant medium:

Half concentrated Murashige and Skoog salts

B5 vitamins

1.5% sucrose

pH 5.8

0.7% Difco agar

Incubation medium:

10mM K-phosphate buffer pH5.8

2% sucrose

1 drop Tween20 for 25ml medium

Reaction buffer:

50mM K-phosphate buffer pH7.4
1mM sodium,3'-{1-[phenylamino-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) = XTT (bts, Germany, cat n° 2525)
1 drop Tween20 for 25ml buffer

[106] *Arabidopsis* plants

- *Arabidopsis* lines: control
  lines to test
- Sterilization of *Arabidopsis* seeds:
  2min. 70% ethanol
  10 min. bleach (6% active chlorine) + 1drop Tween 20 for 20ml solution
  wash 5 times with sterile tap water
- Pregermination of seeds:
  In 9cm Optilux Petridishes (Falcon) containing 12ml sterile tap water.
  Low light overnight to 24 hours.
- Growing of *Arabidopsis* plants
  Seeds are sown in Intergrid Tissue Culture disks of Falcon (nr. 3025) containing ±125ml of plant medium: 1 seed/grid.
  Plants are grown at 24°C
  30µEinstein $s^{-1}m^{-2}$
  16hours light - 8hours dark
  for about 3 weeks (before bolting)

[107] XTT-assay

Control condition (no stress)

- Harvest shoots (roots included) from agar plates and put them directly in a 50ml Falcon tube containing reaction buffer (without XTT)

Stressed shoots

- Transfer shoots to 50ml Falcon tubes containing reaction buffer (without XTT)
- Replace reaction buffer with buffer containing XTT (40mL/tube)

- Shoots have to be submerged, but do not vacuum infiltrate
- Incubate in the dark at 26°C for about 3hours
- Measure the absorption of the reaction medium at 470nm

[108] Quantification of respiration by measuring oxygen consumption using a Clark polarographic electrode was done in the following way:

[109] Plant material

Brassica napus

150-200* hypocotyl explants

Cultured for 5 days at 25°C

(*cfr.* protocol vigour assay)

* 150 explants error <10%; 200 explants error <6%

Arabidopsis

For C24 ± 1000mg* *in vitro* plants (shoots + roots) (corresponds with ~50 18-days old plants)

Pregerminate seeds before sowing

Grow for 18 days at 24°C

(*cfr.* protocol *in vitro* growth Arabidopsis)

* for error <8%

[110] Incubation media

Brassica napus

25mM K-phosphate buffer pH5.8

2% sucrose

Tween20 (1 drop/25ml)

Arabidopsis

10mM K-phosphate buffer pH5.8

2% sucrose

Tween20 (1 drop/25ml)

Before use, aerate (saturate with oxygen) medium well by stirring for at least a few hours

[111] Assay

- Put explants in 100ml glass bottle (Schott, Germany) filled with incubation medium. Put the same weight of shoots in each bottle (± 700mg)
- Fill bottle to overflowing and close tightly (avoid large air bubbles)
- Fill also a bottle with incubation medium that does not contain explants (blanco)
- Incubate at 24°C at low light for: 3-4 hours (*Brassica napus*)
  3 hours (*Arabidopsis*)
- Shake gently during incubation (to avoid oxygen depletion of medium around explants)
- Measure oxygen concentration (mg/l) of incubation media using an hand-held dissolved oxygen meter (Cyberscan DO 310; Eutech Instruments, Singapore)
- mg/l consumed oxygen = [oxygen] blanco – [oxygen] sample.

1. A method to produce a plant tolerant to stress conditions comprising the steps of

(a) providing plant cells with a chimeric gene to create transgenic plant cells, said chimeric gene comprising the following operably linked DNA fragments

(i) a plant-expressible promoter;

(ii) a DNA region, which when transcribed yields an ParG inhibitory RNA molecule;

(iii) a 3' end region involved in transcription termination and polyadenylation;

(b) regenerating a population of transgenic plant lines from said transgenic plant cell; and

(c) identifying a stress tolerant plant line within said population of transgenic plant lines.

2. The method according to claim 1, wherein said parG inhibitory RNA molecule comprises comprising a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in said plant cell.

3. The method according to claim 1, wherein said parG inhibitory RNA molecule is comprises comprising a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in said plant cell.

4. The method according to claim 2 or 3, wherein said chimeric gene further comprises a DNA region encoding a self-splicing ribozyme between said DNA region coding for said parG inhibitory RNA molecule and said 3' end region.

5. The method according to claim 1, wherein said parG inhibitory RNA comprises a sense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in said plant cell and an antisense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide

sequence of the ParG gene present in said plant cell, wherein said sense and antisense region are capable of forming a double stranded RNA region comprising said at least 20 consecutive nucleotides.

6. The method according to any one of claims 1 to 5 wherein said stress conditions is selected from heat, drought, nutrient depletion, oxidative stress or high light conditions.

7. The method according to any one of claims 1 to 6, comprising further crossing said transgenic plant line with another plant line to obtain stress tolerant progeny plants.

8. A method to produce a plant tolerant to stress conditions comprising the steps of:

(a) isolating a DNA fragment of at least 100 bp comprising a part of the parG encoding gene of said plant;

(b) producing a chimeric gene by operably linking the following DNA fragments;

(i) a plant expressible promoter region;

(ii) said isolated DNA fragment comprising part of the parG encoding gene of said plant in direct orientation compared to the promoter region;

(iii) said isolated DNA fragment comprising part of the parG encoding gene of said plant in inverted orientation compared to the promoter region;

(iv) a 3' end region involved in transcription termination and polyadenylation;

(c) providing plant cells with said chimeric gene gene to create transgenic plant cells

(d) regenerating a population of transgenic plant lines from said transgenic plant cell; and

(e) identifying a stress tolerant plant line within said population of transgenic plant lines.

9. A DNA molecule comprising
   (i) a plant-expressible promoter
   (ii) a DNA region, which when transcribed yields an ParG inhibitory RNA molecule
   (iii) a 3' end region involved in transcription termination and polyadenylation, as described in any one of claims 1 to 8.

10. A plant cell comprising the DNA molecule of claims 9.

11. A plant consisting essentially of the plant cells of claim 10.

12. A process for producing stress tolerant plants, comprising the step of further crossing a plant of claim 11 with another plant.

13. Seeds and propagating material of a plant according to claim 10, comprising the chimeric gene of claim 9.

14. Plants obtained by the process of claim 8.

ABSTRACT

Methods and means are provided to increase the tolerance of plants to abiotic stress or adverse growing conditions, including drought, high light intensities, high temperatures, nutrient limitations and the like by reducing the activity of endogenous PARG proteins in plants.

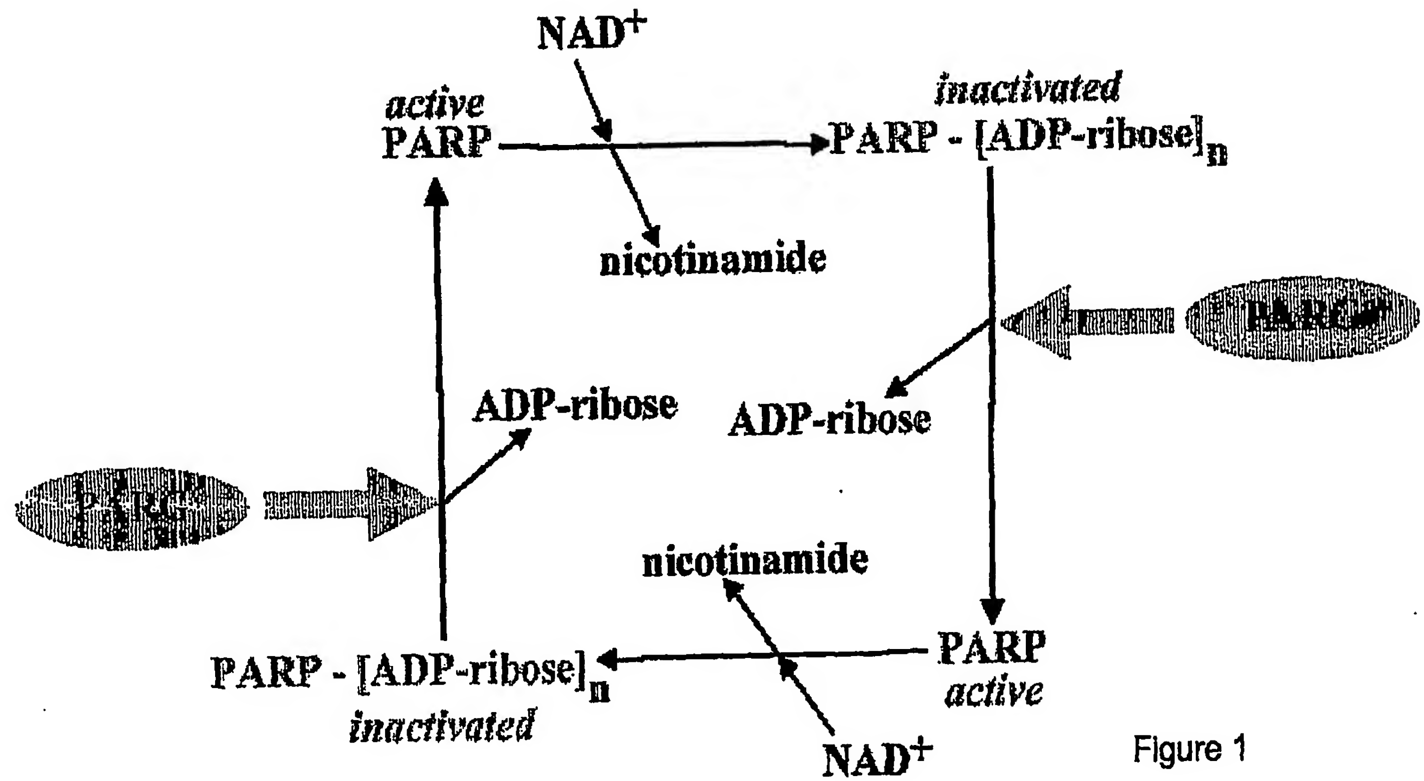
NAD+
active
PARP
inactivated
PARP - [ADP-ribose]n
nicotinamide
ADP-ribose
ADP-ribose
nicotinamide
PARP - [ADP-ribose]n
inactivated
PARP
active
NAD+

Figure 1

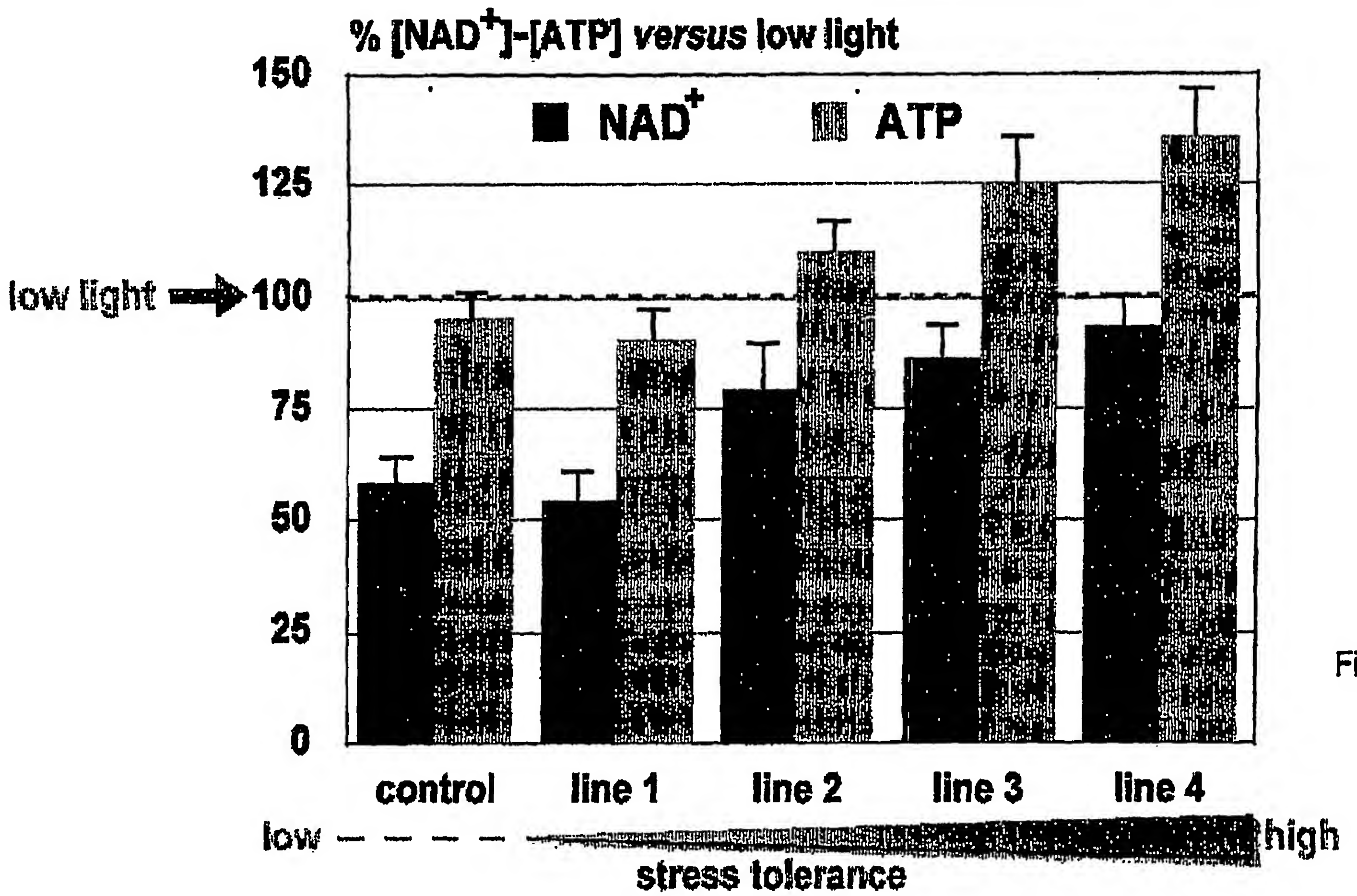
% [NAD+]-[ATP] versus low light
NAD+
ATP
150
125
low light
100
75
50
25
0
control
line 1
line 2
line 3
line 4
low
high
stress tolerance

Figure 2

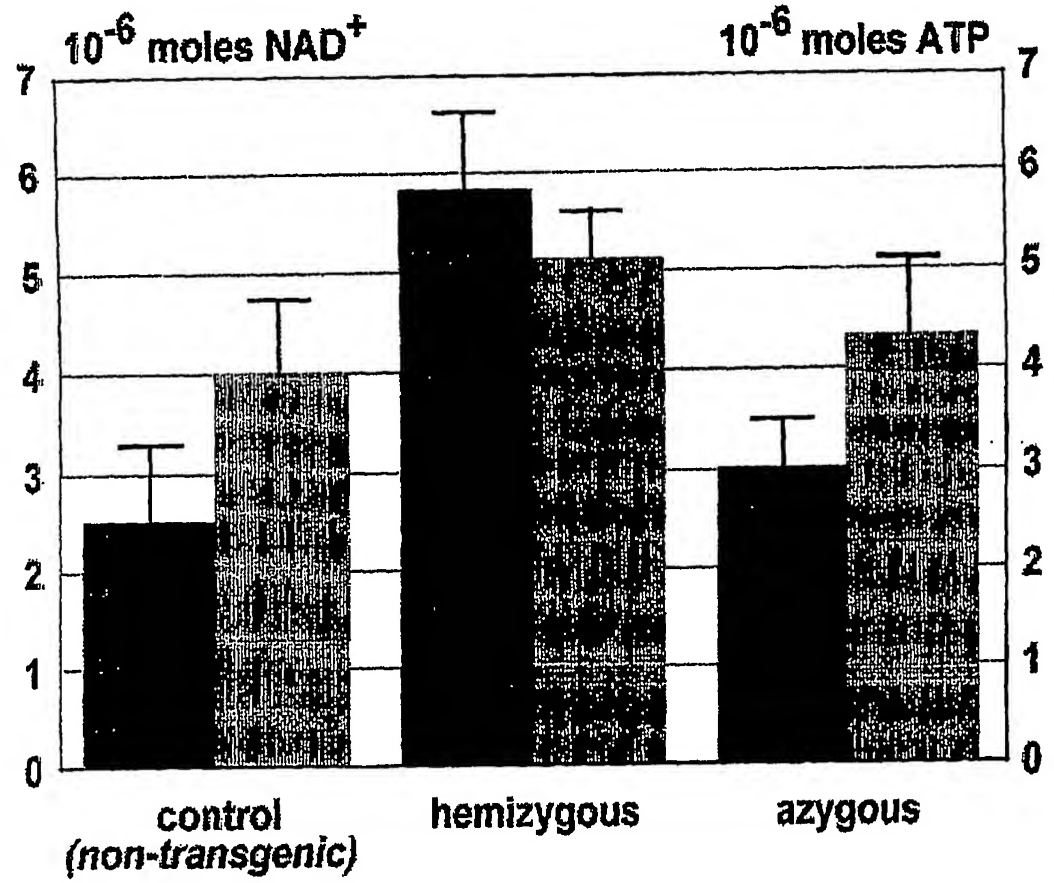
10-6 moles NAD+
10-6 moles ATP
7
6
5
4
3
2
1
0
control
(non-transgenic)
hemizygous
azygous

Figure 3

SEQUENCE LISTING

<110> De Block, Marc

<120> Methods and means for increasing the tolerance of plants to stress conditions.

<130> BCS 03 2002

<160> 14

<170> PatentIn version 3.0

<210> 1

<211> 548

<212> PRT

<213> Arabidopsis thaliana

<400> 1

```
Met Glu Asn Arg Glu Asp Leu Asn Ser Ile Leu Pro Tyr Leu Pro Leu
1               5                   10                  15

Val Ile Arg Ser Ser Ser Leu Tyr Trp Pro Pro Arg Val Val Glu Ala
            20                  25                  30

Leu Lys Ala Met Ser Glu Gly Pro Ser His Ser Gln Val Asp Ser Gly
        35                  40                  45

Glu Val Leu Arg Gln Ala Ile Phe Asp Met Arg Arg Ser Leu Ser Phe
    50                  55                  60

Ser Thr Leu Glu Pro Ser Ala Ser Asn Gly Tyr Ala Phe Leu Phe Asp
65                  70                  75                  80

Glu Leu Ile Asp Glu Lys Glu Ser Lys Arg Trp Phe Asp Glu Ile Ile
                85                  90                  95

Pro Ala Leu Ala Ser Leu Leu Leu Gln Phe Pro Ser Leu Leu Glu Val
            100                 105                 110
```

His Phe Gln Asn Ala Asp Asn Ile Val Ser Gly Ile Lys Thr Gly Leu
115 120 125

Arg Leu Leu Asn Ser Gln Gln Ala Gly Ile Val Phe Leu Ser Gln Glu
130 135 140

Leu Ile Gly Ala Leu Leu Ala Cys Ser Phe Phe Cys Leu Phe Pro Asp
145 150 155 160

Asp Asn Arg Gly Ala Lys His Leu Pro Val Ile Asn Phe Asp His Leu
165 170 175

Phe Ala Ser Leu Tyr Ile Ser Tyr Ser Gln Ser Gln Glu Ser Lys Ile
180 185 190

Arg Cys Ile Met His Tyr Phe Glu Arg Phe Cys Ser Cys Val Pro Ile
195 200 205

Gly Ile Val Ser Phe Glu Arg Lys Ile Thr Ala Ala Pro Asp Ala Asp
210 215 220

Phe Trp Ser Lys Ser Asp Val Ser Leu Cys Ala Phe Lys Val His Ser
225 230 235 240

Phe Gly Leu Ile Glu Asp Gln Pro Asp Asn Ala Leu Glu Val Asp Phe
245 250 255

Ala Asn Lys Tyr Leu Gly Gly Gly Ser Leu Ser Arg Gly Cys Val Gln
260 265 270

Glu Glu Ile Arg Phe Met Ile Asn Pro Glu Leu Ile Ala Gly Met Leu
275 280 285

Phe Leu Pro Arg Met Asp Asp Asn Glu Ala Ile Glu Ile Val Gly Ala
290 295 300

Glu Arg Phe Ser Cys Tyr Thr Gly Tyr Ala Ser Ser Phe Arg Phe Ala
305 310 315 320

Gly Glu Tyr Ile Asp Lys Lys Ala Met Asp Pro Phe Lys Arg Arg Arg
325 330 335

Thr Arg Ile Val Ala Ile Asp Ala Leu Cys Thr Pro Lys Met Arg His
340 345 350

Phe Lys Asp Ile Cys Leu Leu Arg Glu Ile Asn Lys Ala Leu Cys Gly
355 360 365

Phe Leu Asn Cys Ser Lys Ala Trp Glu His Gln Asn Ile Phe Met Asp
370 375 380

Glu Gly Asp Asn Glu Ile Gln Leu Val Arg Asn Gly Arg Asp Ser Gly
385 390 395 400

Leu Leu Arg Thr Glu Thr Thr Ala Ser His Arg Thr Pro Leu Asn Asp
405 410 415

Val Glu Met Asn Arg Glu Lys Pro Ala Asn Asn Leu Ile Arg Asp Phe

420 425 430

Tyr Val Glu Gly Val Asp Asn Glu Asp His Glu Asp Asp Gly Val Ala
435 440 445

Thr Gly Asn Trp Gly Cys Gly Val Phe Gly Gly Asp Pro Glu Leu Lys
450 455 460

Ala Thr Ile Gln Trp Leu Ala Ala Ser Gln Thr Arg Arg Pro Phe Ile
465 470 475 480

Ser Tyr Tyr Thr Phe Gly Val Glu Ala Leu Arg Asn Leu Asp Gln Val
485 490 495

Thr Lys Trp Ile Leu Ser His Lys Trp Thr Val Gly Asp Leu Trp Asn
500 505 510

Met Met Leu Glu Tyr Ser Ala Gln Arg Leu Tyr Lys Gln Thr Ser Val
515 520 525

Gly Phe Phe Ser Trp Leu Leu Pro Ser Leu Ala Thr Thr Asn Lys Ala
530 535 540

Ile Gln Pro Pro
545

<210> 2

<211> 169

<212> PRT

<213> Solanum tuberosum

<400> 2

Met Glu Asn Arg Glu Asp Val Lys Ser Ile Leu Pro Phe Leu Pro Val
1 5 10 15

Cys Leu Arg Ser Ser Ser Leu Phe Trp Pro Pro Leu Val Val Glu Ala
20 25 30

Leu Lys Ala Leu Ser Glu Gly Pro His Tyr Ser Asn Val Asn Ser Gly
35 40 45

Gln Val Leu Phe Leu Ala Ile Ser Asp Ile Arg Asn Ser Leu Ser Leu
50 55 60

Pro Asp Ser Ser Ile Ser Ser Ser Ala Ser Asp Gly Phe Ser Leu Leu
65 70 75 80

Phe Asp Asp Leu Ile Pro Arg Asp Glu Ala Val Lys Trp Phe Lys Glu
85 90 95

Val Val Pro Lys Met Ala Asp Leu Leu Leu Arg Leu Pro Ser Leu Leu
100 105 110

Glu Ala His Tyr Glu Lys Ala Asp Gly Gly Ile Val Lys Gly Val Asn
115 120 125

Thr Gly Leu Arg Leu Leu Glu Ser Gln Gln Pro Gly Ile Val Phe Leu
130 135 140

Ser Gln Glu Leu Val Gly Ala Leu Leu Ala Cys Ser Phe Phe Cys Tyr
145 150 155 160

Ser Leu Pro Met Ile Glu Val Ser Val
165

<210> 3

<211> 1647

<212> DNA

<213> Arabidopsis thaliana

<400> 3
atggagaatc gcgaagatct taactcaatt cttccgtacc ttccacttgt aattcgttcg 60

tcgtcgctgt attggccgcc gcgtgtggtg gaggcgttaa aggcaatgtc tgaaggacca 120

tctcacagcc aagttgactc aggagaggtt ctacggcaag ctattttcga tatgagacga 180

tccttatctt tctctactct cgagccatct gcttctaatg gctacgcatt tctctttgac 240

gaattgattg atgagaaaga atcaaagaga tggttcgatg agattatccc agcattggcg 300

agcttacttc tacagtttcc atctctgtta gaagtgcatt tccaaaatgc tgataatatt 360

gttagtggaa tcaaaaccgg tcttcgtttg ttaaattccc aacaagctgg cattgttttc 420

ctcagccagg agttgattgg agctcttctt gcatgctctt tcttttgttt gtttccggat 480

gataatagag gtgcaaaaca ccttccagtc atcaactttg atcatttgtt tgcaagcctt 540

tatataagtt atagtcaaag tcaagaaagc aagataagat gtattatgca ttactttgaa 600

aggttttgct cctgcgtgcc tattggtatt gtttcatttg aacgcaagat taccgctgct 660

cctgatgctg atttctggag caagtctgac gtttctcttt gtgcatttaa ggttcactct 720

tttgggttaa ttgaagatca acctgacaat gctctcgaag tggactttgc aaacaagtat 780

ctcggaggtg gttccctaag tagagggtgc gtgcaggaag agatacgctt catgattaac 840

cctgaattaa tcgctggcat gcttttcttg cctcggatgg atgacaatga agctatagaa 900

atagttggtg cggaaagatt ttcatgttac acagggtatg catcttcgtt tcggtttgct 960

ggtgagtaca ttgacaaaaa ggcaatggat cctttcaaaa ggcgaagaac cagaattgtt 1020

gcaattgatg cattatgtac accgaagatg agacacttta aagatatatg tcttttaagg 1080
gaaattaata aggcactatg tggcttttta aattgtagca aggcttggga gcaccagaat 1140
atattcatgg atgaaggaga taatgaaatt cagcttgtcc gaaacggcag agattctggt 1200
cttctgcgta cagaaactac tgcgtcacac cgaactccac taaatgatgt tgagatgaat 1260
agagaaaagc ctgctaacaa tcttatcaga gatttttatg tggaaggagt tgataacgag 1320
gatcatgaag atgatggtgt cgcgacaggg aattggggat gtggtgtttt tggaggagac 1380
ccagagctaa aggctacgat acaatggctt gctgcttccc agactcgaag accatttata 1440
tcatattaca cctttggagt agaggcactc cgaaacctag atcaggtgac gaagtggatt 1500
ctttcccata aatggactgt tggagatctg tggaacatga tgttagaata ttctgctcaa 1560
aggctctaca agcaaaccag tgttggcttc ttttcttggc tacttccatc tctagctacc 1620
accaacaaag ctatccagcc gccttga 1647

<210> 4

<211> 598

<212> DNA

<213> Solanum tuberosum

<400> 4
gcaatggaga atagagaaga cgtgaagtca atccttccct ttttgccggt gtgtctccga 60
tcatcttctc ttttctggcc gccgctagtt gttgaagcac tgaaagccct ctctgaaggc 120
cctcattaca gcaatgttaa ctccggccaa gtcctcttcc tcgcaatctc cgacattcgg 180
aattcccttt cactacctga ttcttcaatt tcctcttctg cttcagacgg attttctctc 240
ttatttgaty atttaattcc tagggatgaa gctgttaaat ggttcaaaga agtggtgccg 300
aaaatggcgg atttgctatt gcggttgcct tccttattgg aggctcacta tgagaaggct 360
gatggtggaa ttgttaaagg agtcaacact ggtcttcgct tattggaatc acaacagcct 420
ggcattgttt tcctcagtca ggaattagtc ggtgctcttc ttgcatgttc cttcttttgc 480
tattccctac caatgataga ggtatctgta tgatcagtat gacgagaaat ttgaaaataa 540
attgaagtgc attcttcact attttgagag gattggctca ttgatacctg cgggctac 598

<210> 5

<211> 37

<212> DNA

<213> Artificial

<220>

<223> oligonucleotide primer ParGAt1

<400> 5
ggatcccctg caggacaaaa aggcaatgga tcctttc 37

<210> 6

<211> 39

<212> DNA

<213> Artificial

<220>

<223> oligonucleotide primer ParGAt2

<400> 6
gcacgaattc gcggccgcgg tgctcccaag ccttgctac 39

<210> 7

<211> 39

<212> DNA

<213> Artificial

<220>

<223> oligonucleotide primer ParGSt1

<400> 7
ggatcccctg caggctcact atgagaaggc tgatggtgg 39

<210> 8

<211> 43

<212> DNA

<213> Artificial

<220>

<223> oligonucleotide primer ParGSt2

<400> 8
gcacgaattc gcggccgcgt catactgatc atacagatac ctc 43

<210> 9

<211> 13466

<212> DNA

<213> Artificial

<220>

<223> nucleotide sequence of pTVE428

<220>

<221> misc_feature

<222> (198)..(222)

<223> Right T-DNA border

<220>

<221> misc_feature

<222> (983)..(273)

<223> 3' ocs (3' untranslated end of octopine synthase gene)

<220>

<221> misc_feature

<222> (995)..(1155)

<223> part of poly (ADP-ribose) glycohydrolase

<220>

<221> misc_feature

<222> (1929)..(1188)

<223> intron 2 from the Pdk gene of Flaveria

<220>

<221> misc_feature

<222> (2122)..(1962)

<223> part of poly (ADP-ribose) glycohydrolase

<220>

<221> misc_feature

<222> (3476)..(2131)

<223> 35S promoter region from Cauliflower Mosaic Virus

<220>

<221> misc_feature

<222> (3948)..(3737)

<223> 3' untranslated end of gene 7 from Agrobacterium tumefaciens

<220>

<221> misc_feature

<222> (4521)..(3970)

<223> bar coding region

<220>

<221> misc_feature

<222> (6247)..(4522)

<223> PSSuAra promoter region

<220>

<221> misc_feature

<222> (6415)..(6439)

<223> left border region of T-DNA of Agrobacterium tumefaciens

<400> 9
agattcgaag ctcggtcccg tgggtgttct gtcgtctcgt tgtacaacga aatccattcc 60

cattccgcgc tcaagatggc ttcccctcgg cagttcatca gggctaaatc aatctagccg 120

acttgtccgg tgaaatgggc tgcactccaa cagaaacaat caaacaaaca tacacagcga 180

cttattcaca cgcgacaaat tacaacggta tatatcctgc cagtactcgg ccgtcgaccg 240

cggtaccccg gaattaagct tgcatgcctg caggtcctgc tgagcctcga catgttgtcg 300

caaaattcgc cctggacccg cccaacgatt tgtcgtcact gtcaaggttt gacctgcact 360

tcatttgggg cccacataca ccaaaaaaat gctgcataat tctcggggca gcaagtcggt 420

tacccggccg ccgtgctgga ccgggttgaa tggtgcccgt aactttcggt agagcggacg 480

gccaatactc aacttcaagg aatctcaccc atgcgcgccg gcggggaacc ggagttccct 540

tcagtgaacg ttattagttc gccgctcggt gtgtcgtaga tactagcccc tggggccttt 600

tgaaatttga ataagattta tgtaatcagt cttttaggtt tgaccggttc tgccgctttt 660

tttaaaattg gatttgtaat aataaaacgc aattgtttgt tattgtggcg ctctatcata 720

gatgtcgcta taaacctatt cagcacaata tattgttttc attttaatat tgtacatata 780

agtagtaggg tacaatcagt aaattgaacg gagaatatta ttcataaaaa tacgatagta 840

acgggtgata tattcattag aatgaaccga aaccggcggt aaggatctga gctacacatg 900

ctcaggtttt ttacaacgtg cacaacagaa ttgaaagcaa atatcatgcg atcataggcg 960

tctcgcatat ctcattaaag caggactcta gagacaaaaa ggcaatggat cctttcaaaa 1020

ggcgaagaac cagaattgtt gcaattgatg cattatgtac accgaagatg agacacttta 1080

aagatatatg tcttttaagg gaaattaata aggcactatg tggcttttta aattgtagca 1140

aggcttggga gcaccatcga tttcgaaccc agcttcccaa ctgtaatcaa tccaaatgta 1200

agatcaatga taacacaatg acatgatcta tcatgttacc ttgtttattc atgttcgact 1260

aattcattta attaatagtc aatccattta gaagttaata aaactacaag tattatttag 1320

aaattaataa gaatgttgat tgaaaataat actatataaa atgatagatc ttgcgctttg 1380

ttatattagc attagattat gttttgttac attagattac tgtttctatt agtttgatat 1440

tatttgttac tttagcttgt tatttaatat tttgtttatt gataaattac aagcagattg 1500
gaatttctaa caaaatattt attaactttt aaactaaaat atttagtaat ggtatagata 1560
tttaattata taataaacta ttaatcataa aaaaatatta ttttaattta tttattctta 1620
tttttactat agtattttat cattgatatt taattcatca aaccagctag aattactatt 1680
atgattaaaa caaatattaa tgctagtata tcatcttaca tgttcgatca aattcattaa 1740
aaataatata cttactctca acttttatct tcttcgtctt acacatcact tgtcatattt 1800
ttttacatta ctatgttgtt tatgtaaaca atatatttat aaattatttt ttcacaatta 1860
taacaactat attattataa tcatactaat taacatcact taactatttt atactaaaag 1920
gaaaaaagaa aataattatt tccttaccaa gctggggtac cggtgctccc aagccttgct 1980
acaatttaaa aagccacata gtgccttatt aatttccctt aaaagacata tatctttaaa 2040
gtgtctcatc ttcggtgtac ataatgcatc aattgcaaca attctggttc ttcgcctttt 2100
gaaaggatcc attgcctttt tgtcctcgag cgtgtcctct ccaaatgaaa tgaacttcct 2160
tatatagagg aagggtcttg cgaaggatag tgggattgtg cgtcatccct tacgtcagtg 2220
gagatgtcac atcaatccac ttgctttgaa gacgtggttg gaacgtcttc tttttccacg 2280
atgctcctcg tgggtggggg tccatctttg ggaccactgt cggcagagag atcttgaatg 2340
atagcctttc ctttatcgca atgatggcat ttgtaggagc caccttcctt ttctactgtc 2400
ctttcgatga agtgacagat agctgggcaa tggaatccga ggaggtttcc cgaaattatc 2460
ctttgttgaa aagtctcaat agccctttgg tcttctgaga ctgtatcttt gacatttttg 2520
gagtagacca gagtgtcgtg ctccaccatg ttgacgaaga ttttcttctt gtcattgagt 2580
cgtaaaagac tctgtatgaa ctgttcgcca gtcttcacgg cgagttctgt tagatcctcg 2640
atttgaatct tagactccat gcatggcctt agattcagta ggaactacct ttttagagac 2700
tccaatctct attacttgcc ttggtttatg aagcaagcct tgaatcgtcc atactggaat 2760
agtacttctg atcttgagaa atatgtcttt ctctgtgttc ttgatgcaat tagtcctgaa 2820
tcttttgact gcatctttaa ccttcttggg aaggtatttg atctcctgga gattgttact 2880
cgggtagatc gtcttgatga gacctgctgc gtaggcctct ctaaccatct gtgggtcagc 2940
attctttctg aaattgaaga ggctaacctt ctcattatca gtggtgaaca tagtgtcgtc 3000
accttcacct tcgaacttcc ttcctagatc gtaaagatag aggaaatcgt ccattgtaat 3060
ctccggggca aaggagatct cttttggggc tggatcactg ctgggccttt tggttcctag 3120
cgtgagccag tgggcttttt gctttggtgg gcttgttagg gccttagcaa agctcttggg 3180

cttgagttga gcttctcctt tggggatgaa gttcaacctg tctgtttgct gacttgttgt 3240
gtacgcgtca gctgctgctc ttgcctctgt aatagtggca aatttcttgt gtgcaactcc 3300
gggaacgccg tttgttgccg cctttgtaca accccagtca tcgtatatac cggcatgtgg 3360
accgttatac acaacgtagt agttgatatg agggtgttga atacccgatt ctgctctgag 3420
aggagcaact gtgctgttaa gctcagattt ttgtgggatt ggaattaatt cgtcgagcgg 3480
ccgctcgacg agcgcgccga tatcgcgatc gcccgggccg gccatttaaa tgaattcgag 3540
ctcggtaccc aaacgcggcc gcaagctata acttcgtata gcatacatta tacgaagtta 3600
ttcgactcta gaggatccca attcccatgc atggagtcaa agattcaaat agaggacact 3660
tctcgaactc ggccgtcgaa ctcggccgtc gagtacatgg tcgataagaa aaggcaattt 3720
gtagatgtta attcccatct tgaaagaaat atagtttaaa tatttattga taaaataaca 3780
agtcaggtat tatagtccaa gcaaaaacat aaatttattg atgcaagttt aaattcagaa 3840
atatttcaat aactgattat atcagctggt acattgccgt agatgaaaga ctgagtgcga 3900
tattatgtgt aatacataaa ttgatgatat agctagctta gctcatcggg ggatcctaga 3960
cgcgtgagat cagatctcgg tgacgggcag gaccggacgg ggcggtaccg gcaggctgaa 4020
gtccagctgc cagaaaccca cgtcatgcca gttcccgtgc ttgaagccgg ccgcccgcag 4080
catgccgcgg ggggcatatc cgagcgcctc gtgcatgcgc acgctcgggt cgttgggcag 4140
cccgatgaca gcgaccacgc tcttgaagcc ctgtgcctcc agggacttca gcaggtgggt 4200
gtagagcgtg gagcccagtc ccgtccgctg gtggcggggg gagacgtaca cggtcgactc 4260
ggccgtccag tcgtaggcgt tgcgtgcctt ccaggggccc gcgtaggcga tgccggcgac 4320
ctcgccgtcc acctcggcga cgagccaggg atagcgctcc cgcagacgga cgaggtcgtc 4380
cgtccactcc tgcggttcct gcggctcggt acggaagttg accgtgcttg tctcgatgta 4440
gtggttgacg atggtgcaga ccgccggcat gtccgcctcg gtggcacggc ggatgtcggc 4500
cgggcgtcgt tctgggtcca ttgttcttct ttactctttg tgtgactgag gtttggtcta 4560
gtgctttggt catctatata taatgataac aacaatgaga acaagctttg gagtgatcgg 4620
agggtctagg atacatgaga ttcaagtgga ctaggatcta caccgttgga ttttgagtgt 4680
ggatatgtgt gaggttaatt ttacttggta acggccacaa aggcctaagg agaggtgttg 4740
agacccttat cggcttgaac cgctggaata atgccacgtg gaagataatt ccatgaatct 4800
tatcgttatc tatgagtgaa attgtgtgat ggtggagtgg tgcttgctca ttttacttgc 4860
ctggtggact tggccctttc cttatgggga atttatattt tacttactat agagctttca 4920

taccttttttt ttaccttgga tttagttaat atataatggt atgattcatg aataaaaatg 4980
ggaaattttt gaatttgtac tgctaaatgc ataagattag gtgaaactgt ggaatatata 5040
tttttttcat ttaaaagcaa aatttgcctt ttactagaat tataaatata gaaaaatata 5100
taacattcaa ataaaaatga aaataagaac tttcaaaaaa cagaactatg tttaatgtgt 5160
aaagattagt cgcacatcaa gtcatctgtt acaatatgtt acaacaagtc ataagcccaa 5220
caaagttagc acgtctaaat aaactaaaga gtccacgaaa atattacaaa tcataagccc 5280
aacaaagtta ttgatcaaaa aaaaaaaacg cccaacaaag ctaaacaaag tccaaaaaaa 5340
acttctcaag tctccatctt cctttatgaa cattgaaaac tatacacaaa acaagtcaga 5400
taaatctctt tctgggcctg tcttcccaac ctcctacatc acttccctat cggattgaat 5460
gttttacttg taccttttcc gttgcaatga tattgatagt atgtttgtga aaactaatag 5520
ggttaacaat cgaagtcatg gaatatggat ttggtccaag attttccgag agctttctag 5580
tagaaagccc atcaccagaa atttactagt aaaataaatc accaattagg tttcttatta 5640
tgtgccaaat tcaatataat tatagaggat atttcaaatg aaaacgtatg aatgttatta 5700
gtaaatggtc aggtaagaca ttaaaaaaat cctacgtcag atattcaact ttaaaaattc 5760
gatcagtgtg gaattgtaca aaaatttggg atctactata tatatataat gctttacaac 5820
acttggattt ttttttggag gctggaattt ttaatctaca tatttgtttt ggccatgcac 5880
caactcattg tttagtgtaa tactttgatt ttgtcaaata tatgtgttcg tgtatatttg 5940
tataagaatt tctttgacca tatacacaca cacatatata tatatatata tatattatat 6000
atcatgcact tttaattgaa aaaataatat atatatatat agtgcatttt ttctaacaac 6060
catatatgtt gcgattgatc tgcaaaaata ctgctagagt aatgaaaaat ataatctatt 6120
gctgaaatta tctcagatgt taagattttc ttaaagtaaa ttctttcaaa ttttagctaa 6180
aagtcttgta ataactaaag aataatacac aatctcgacc acggaaaaaa aacacataat 6240
aaatttgaat ttcgaccgcg gtacccggaa ttgggttata attacctcag gtcgaggaat 6300
taattcggta cgtacctaat aacttcgtat agcatacatt atacgaagtt atatggatct 6360
cgaggcatta cggcattacg gcactcgcga gggtcccaat tcgagcatgg agccatttac 6420
aattgaatat atcctgccgc cgctgccgct ttgcacccgg tggagcttgc atgttggttt 6480
ctacgcagaa ctgagccggt taggcagata atttccattg agaactgagc catgtgcacc 6540
ttcccccccaa cacggtgagc gacggggcaa cggagtgatc cacatgggac ttttaaacat 6600
catccgtcgg atggcgttgc gagagaagca gtcgatccgt gagatcagcc gacgcaccgg 6660

gcaggcgcgc aacacgatcg caaagtattt gaacgcaggt acaatcgagc cgacgttcac 6720
ggtaccggaa cgaccaagca agctagctta gtaaagccct cgctagattt taatgcggat 6780
gttgcgatta cttcgccaac tattgcgata acaagaaaaa gccagccttt catgatatat 6840
ctcccaattt gtgtagggct tattatgcac gcttaaaaat aataaaagca gacttgacct 6900
gatagtttgg ctgtgagcaa ttatgtgctt agtgcatcta acgcttgagt taagccgcgc 6960
cgcgaagcgg cgtcggcttg aacgaattgt tagacattat ttgccgacta ccttggtgat 7020
ctcgcctttc acgtagtgga caaattcttc caactgatct gcgcgcgagg ccaagcgatc 7080
ttcttcttgt ccaagataag cctgtctagc ttcaagtatg acgggctgat actgggccgg 7140
caggcgctcc attgcccagt cggcagcgac atccttcggc gcgattttgc cggttactgc 7200
gctgtaccaa atgcgggaca acgtaagcac tacatttcgc tcatcgccag cccagtcggg 7260
cggcgagttc catagcgtta aggtttcatt tagcgcctca aatagatcct gttcaggaac 7320
cggatcaaag agttcctccg ccgctggacc taccaaggca acgctatgtt ctcttgcttt 7380
tgtcagcaag atagccagat caatgtcgat cgtggctggc tcgaagatac ctgcaagaat 7440
gtcattgcgc tgccattctc caaattgcag ttcgcgctta gctggataac gccacggaat 7500
gatgtcgtcg tgcacaacaa tggtgacttc tacagcgcgg agaatctcgc tctctccagg 7560
ggaagccgaa gtttccaaaa ggtcgttgat caaagctcgc cgcgttgttt catcaagcct 7620
tacggtcacc gtaaccagca aatcaatatc actgtgtggc ttcaggccgc catccactgc 7680
ggagccgtac aaatgtacgg ccagcaacgt cggttcgaga tggcgctcga tgacgccaac 7740
tacctctgat agttgagtcg atacttcggc gatcaccgct tccctcatga tgtttaactt 7800
tgttttaggg cgactgccct gctgcgtaac atcgttgctg ctccataaca tcaaacatcg 7860
acccacggcg taacgcgctt gctgcttgga tgcccgaggc atagactgta ccccaaaaaa 7920
acagtcataa caagccatga aaaccgccac tgcgccgtta ccaccgctgc gttcggtcaa 7980
ggttctggac cagttgcgtg agcgcatacg ctacttgcat tacagcttac gaaccgaaca 8040
ggcttatgtc cactgggttc gtgccttcat ccgtttccac ggtgtgcgtc acccggcaac 8100
cttgggcagc agcgaagtcg aggcatttct gtcctggctg gcgaacgagc gcaaggtttc 8160
ggtctccacg catcgtcagg cattggcggc cttgctgttc ttctacggca agtgctgtgc 8220
acggatctgc cctggcttca ggagatcgga agacctcggc cgtccgggcg cttgccggtg 8280
gtgctgaccc cggatgaagt ctctagagct ctagagggtt cgcatcctcg gttttctgga 8340
aggcgagcat cgtttgttcg cccagcttct gtatggaacg ggcatgcgga tcagtgaggg 8400

tttgcaactg cgggtcaagg atctggattt cgatcacggc acgatcatcg tgcgggaggg 8460
caagggctcc aaggatcggg ccttgatgtt acccgagagc ttggcaccca gcctgcgcga 8520
gcagggatcg atccaacccc tccgctgcta tagtgcagtc ggcttctgac gttcagtgca 8580
gccgtcttct gaaaacgaca tgtcgcacaa gtcctaagtt acgcgacagg ctgccgccct 8640
gcccttttcc tggcgttttc ttgtcgcgtg ttttagtcgc ataaagtaga atacttgcga 8700
ctagaaccgg agacattacg ccatgaacaa gagcgccgcc gctggcctgc tgggctatgc 8760
ccgcgtcagc accgacgacc aggacttgac caaccaacgg gccgaactgc acgcggccgg 8820
ctgcaccaag ctgttttccg agaagatcac cggcaccagg cgcgaccgcc cggagctggc 8880
caggatgctt gaccacctac gccctggcga cgttgtgaca gtgaccaggc tagaccgcct 8940
ggcccgcagc acccgcgacc tactggacat tgccgagcgc atccaggagg ccggcgcggg 9000
cctgcgtagc ctggcagagc cgtgggccga caccaccacg ccggccggcc gcatggtgtt 9060
gaccgtgttc gccggcattg ccgagttcga gcgttcccta atcatcgacc gcacccggag 9120
cgggcgcgag gccgccaagg cccgaggcgt gaagtttggc ccccgcccta ccctcacccc 9180
ggcacagatc gcgcacgccc gcgagctgat cgaccaggaa ggccgcaccg tgaaagaggc 9240
ggctgcactg cttggcgtgc atcgctcgac cctgtaccgc gcacttgagc gcagcgagga 9300
agtgacgccc accgaggcca ggcggcgcgg tgccttccgt gaggacgcat tgaccgaggc 9360
cgacgccctg gcggccgccg agaatgaacg ccaagaggaa caagcatgaa accgcaccag 9420
gacggccagg acgaaccgtt tttcattacc gaagagatcg aggcggagat gatcgcggcc 9480
gggtacgtgt tcgagccgcc cgcgcacgtc tcaaccgtgc ggctgcatga aatcctggcc 9540
ggtttgtctg atgccaagct ggcggcctgg ccggccagct tggccgctga agaaaccgag 9600
cgccgccgtc taaaaaggtg atgtgtattt gagtaaaaca gcttgcgtca tgcggtcgct 9660
gcgtatatga tgcgatgagt aaataaacaa atacgcaagg ggaacgcatg aaggttatcg 9720
ctgtacttaa ccagaaaggc gggtcaggca agacgaccat cgcaacccat ctagcccgcg 9780
ccctgcaact cgccggggcc gatgttctgt tagtcgattc cgatccccag ggcagtgccc 9840
gcgattgggc ggccgtgcgg gaagatcaac cgctaaccgt tgtcggcatc gaccgcccga 9900
cgattgaccg cgacgtgaag gccatcggcc ggcgcgactt cgtagtgatc gacggagcgc 9960
cccaggcggc ggacttggct gtgtccgcga tcaaggcagc cgacttcgtg ctgattccgg 10020
tgcagccaag cccttacgac atatgggcca ccgccgacct ggtggagctg gttaagcagc 10080
gcattgaggt cacggatgga aggctacaag cggcctttgt cgtgtcgcgg gcgatcaaag 10140

gcacgcgcat cggcggtgag gttgccgagg cgctggccgg gtacgagctg cccattcttg 10200
agtcccgtat cacgcagcgc gtgagctacc caggcactgc cgccgccggc acaaccgttc 10260
ttgaatcaga acccgagggc gacgctgccc gcgaggtcca ggcgctggcc gctgaaatta 10320
aatcaaaact catttgagtt aatgaggtaa agagaaaatg agcaaaagca caaacacgct 10380
aagtgccggc cgtccgagcg cacgcagcag caaggctgca acgttggcca gcctggcaga 10440
cacgccagcc atgaagcggg tcaactttca gttgccggcg gaggatcaca ccaagctgaa 10500
gatgtacgcg gtacgccaag gcaagaccat taccgagctg ctatctgaat acatcgcgca 10560
gctaccagag taaatgagca aatgaataaa tgagtagatg aattttagcg gctaaaggag 10620
gcggcatgga aaatcaagaa caaccaggca ccgacgccgt ggaatgcccc atgtgtggag 10680
gaacgggcgg ttggccaggc gtaagcggct gggttgtctg ccggccctgc aatggcactg 10740
gaacccccaa gcccgaggaa tcggcgtgac ggtcgcaaac catccggccc ggtacaaatc 10800
ggcgcggcgc tgggtgatga cctggtggag aagttgaagg ccgcgcaggc cgcccagcgg 10860
caacgcatcg aggcagaagc acgccccggt gaatcgtggc aagcggccgc tgatcgaatc 10920
cgcaaagaat cccggcaacc gccggcagcc ggtgcgccgt cgattaggaa gccgcccaag 10980
ggcgacgagc aaccagattt tttcgttccg atgctctatg acgtgggcac ccgcgatagt 11040
cgcagcatca tggacgtggc cgttttccgt ctgtcgaagc gtgaccgacg agctggcgag 11100
gtgatccgct acgagcttcc agacgggcac gtagaggttt ccgcagggcc ggccggcatg 11160
gccagtgtgt gggattacga cctggtactg atggcggttt cccatctaac cgaatccatg 11220
aaccgatacc gggaagggaa gggagacaag cccggccgcg tgttccgtcc acacgttgcg 11280
gacgtactca agttctgccg gcgagccgat ggcggaaagc agaaagacga cctggtagaa 11340
acctgcattc ggttaaacac cacgcacgtt gccatgcagc gtacgaagaa ggccaagaac 11400
ggccgcctgg tgacggtatc cgagggtgaa gccttgatta gccgctacaa gatcgtaaag 11460
agcgaaaccg ggcggccgga gtacatcgag atcgagctag ctgattggat gtaccgcgag 11520
atcacagaag gcaagaaccc ggacgtgctg acggttcacc ccgattactt tttgatcgat 11580
cccggcatcg gccgttttct ctaccgcctg gcacgccgcg ccgcaggcaa ggcagaagcc 11640
agatggttgt tcaagacgat ctacgaacgc agtggcagcg ccggagagtt caagaagttc 11700
tgtttcaccg tgcgcaagct gatcgggtca aatgacctgc cggagtacga tttgaaggag 11760
gaggcggggc aggctggccc gatcctagtc atgcgctacc gcaacctgat cgagggcgaa 11820
gcatccgccg gttcctaatg tacggagcag atgctagggc aaattgccct agcaggggaa 11880

aaaggtcgaa aaggtctctt tcctgtggat agcacgtaca ttgggaaccc aaagccgtac 11940
attgggaacc ggaacccgta cattgggaac ccaaagccgt acattgggaa ccggtcacac 12000
atgtaagtga ctgatataaa agagaaaaaa ggcgattttt ccgcctaaaa ctctttaaaa 12060
cttattaaaa ctcttaaaac ccgcctggcc tgtgcataac tgtctggcca gcgcacagcc 12120
gaagagctgc aaaaagcgcc tacccttcgg tcgctgcgct ccctacgccc cgccgcttcg 12180
cgtcggccta tcgcggccgc tggccgctca aaaatggctg gcctacggcc aggcaatcta 12240
ccagggcgcg gacaagccgc gccgtcgcca ctcgaccgcc ggcgcccaca tcaaggcacc 12300
ctgcctcgcg cgtttcggtg atgacggtga aaacctctga cacatgcagc tcccggagac 12360
ggtcacagct tgtctgtaag cggatgccgg gagcagacaa gcccgtcagg gcgcgtcagc 12420
gggtgttggc gggtgtcggg gcgcagccat gacccagtca cgtagcgata gcggagtgta 12480
tactggctta actatgcggc atcagagcag attgtactga gagtgcacca tatgcggtgt 12540
gaaataccgc acagatgcgt aaggagaaaa taccgcatca ggcgctcttc cgcttcctcg 12600
ctcactgact cgctgcgctc ggtcgttcgg ctgcggcgag cggtatcagc tcactcaaag 12660
gcggtaatac ggttatccac agaatcaggg gataacgcag gaaagaacat gtgagcaaaa 12720
ggccagcaaa aggccaggaa ccgtaaaaag gccgcgttgc tggcgttttt ccataggctc 12780
cgcccccctg acgagcatca caaaaatcga cgctcaagtc agaggtggcg aaacccgaca 12840
ggactataaa gataccaggc gtttccccct ggaagctccc tcgtgcgctc tcctgttccg 12900
accctgccgc ttaccggata cctgtccgcc tttctccctt cgggaagcgt ggcgctttct 12960
catagctcac gctgtaggta tctcagttcg gtgtaggtcg ttcgctccaa gctgggctgt 13020
gtgcacgaac cccccgttca gcccgaccgc tgcgccttat ccggtaacta tcgtcttgag 13080
tccaacccgg taagacacga cttatcgcca ctggcagcag ccactggtaa caggattagc 13140
agagcgaggt atgtaggcgg tgctacagag ttcttgaagt ggtggcctaa ctacggctac 13200
actagaagga cagtatttgg tatctgcgct ctgctgaagc cagttacctt cggaaaaaga 13260
gttggtagct cttgatccgg caaacaaacc accgctggta gcggtggttt ttttgtttgc 13320
aagcagcaga ttacgcgcag aaaaaaagga tctcaagaag atcctttgat cttttctacg 13380
aagagaaagc aggtagcttg cagtgggctt acatggcgat agctagactg ggcggtttta 13440
tggacagcaa gcgaaccgga attgcc 13466

<210> 10

<211> 31

<212> PRT

<213> Artificial

<220>

<223> Consensus sequence 1 of PARG protein

<220>

<221> VARIANT

<222> (2)..(2)

<223> X represents any amino acid

<220>

<221> VARIANT

<222> (8)..(10)

<223> X represents any amino acid

<220>

<221> VARIANT

<222> (14)..(17)

<223> X represents any amino acid

<220>

<221> VARIANT

<222> (19)..(19)

<223> X represents any amino acid

<220>

<221> VARIANT

<222> (27)..(29)

<223> X represents any amino acid

<400> 10

Leu Xaa Val Asp Phe Ala Asn Xaa Xaa Xaa Gly Gly Gly Xaa Xaa Xaa
1 5 10 15

Xaa Gly Xaa Val Gln Glu Glu Ile Arg Phe Xaa Xaa Xaa Pro Glu
20 25 30

<210> 11

<211> 20

<212> PRT

<213> Artificial

<220>

<223> Consensus sequence 2 for PARG protein

<220>

<221> VARIANT

<222> (3)..(3)

<223> X represents any amino acid

<220>

<221> VARIANT

<222> (10)..(10)

<223> X represents any amino acid

<220>

<221> VARIANT

<222> (13)..(14)

<223> X represents any amino acid

<220>

<221> VARIANT

<222> (17)..(19)

<223> X represents any amino acid

<400> 11

```
Thr Gly Xaa Trp Gly Cys Gly Ala Phe Xaa Gly Asp Xaa Xaa Leu Lys
1               5                   10                  15

Xaa Xaa Xaa Gln
            20
```

<210> 12

<211> 13

<212> PRT

<213> Artificial

<220>

<223> Consensus sequence 3 for PARG protein

<220>

<221> VARIANT

<222> (2)..(4)

<223> X represents any amino acid

<220>

<221> VARIANT

<222> (6)..(9)

<223> X represents any amino acid

<400> 12

```
Asp Xaa Xaa Xaa Arg Xaa Xaa Xaa Xaa Ala Ile Asp Ala
1               5                   10
```

<210> 13

<211> 10

<212> PRT

<213> Artificial

<220>

<223> Consensus sequence 4 for PARG protein

<220>

<221> VARIANT

<222> (3)..(4)

<223> X represents any amino acid

<220>

<221> VARIANT

<222> (7)..(8)

<223> X represents any amino acid

<400> 13

Arg Glu Xaa Xaa Lys Ala Xaa Xaa Gly Phe
1 5 10

<210> 14

<211> 11

<212> PRT

<213> Artificial

<220>

<223> conserved PARG region

<220>

<221> VARIANT

<222> (2)..(5)

<223> X represents any amino acid

<220>

<221> VARIANT

<222> (2)..(5)

<223> X represents any amino acid

<400> 14

Gly Xaa Xaa Xaa Xaa Ser Xaa Tyr Thr Gly Tyr
1 5 10

This Page is inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☑ BLURED OR ILLEGIBLE TEXT OR DRAWING
- ☑ SKEWED/SLANTED IMAGES
- ☐ COLORED OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☑ REPERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: ______________________

IMAGES ARE BEST AVAILABLE COPY.
As rescanning documents *will not* correct images problems checked, please do not report the problems to the IFW Image Problem Mailbox